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Searching for Genes Predisposing to Common Dyslipidemias

Department of Molecular Medicine
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SEARCHING FOR GENES PREDISPOSING TO COMMON DYSLIPIDEMIAS

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Academic Dissertation

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Twenty years from now
you will be more disappointed
by the things that you didn't do
than by the ones you did do.
So throw off the bowlines.
Sail away from the safe harbor.
Catch the trade winds in your sails.
Explore. Dream. Discover.

Mark Twain

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals (I-IV):

I Heidi E. Lilja, Aino Soro, Kati Ylitalo, Ilpo Nuotio, Jorma S.A. Viikari, Veikko Salomaa, Erkki Vartiainen, Marja-Riitta Taskinen, Leena Peltonen, Päivi Pajukanta: A candidate gene study in low HDL-cholesterol provides evidence for the involvement of the APOA2 gene and the APOA1C3A4 gene cluster. *Atherosclerosis* 2002, 164:103-111.

II Aino Soro*, Päivi Pajukanta*, Heidi E. Lilja, Kati Ylitalo, Tero Hiekkalinna, Markus Perola, Rita M. Cantor, Jorma S.A. Viikari, Marja-Riitta Taskinen, Leena Peltonen: Genome scans provide evidence for low-HDL-C loci on chromosomes 8q23, 16q24.1-24.2, and 20q13.11 in Finnish families. *The American Journal of Human Genetics* 2002, 70:1333-1340.

III Heidi E. Lilja, Elina Suviolahti, Aino Soro-Paavonen, Tero Hiekkalinna, Aaron Day, Kenneth Lange, Eric Sobel, Marja-Riitta Taskinen, Leena Peltonen, Markus Perola, and Päivi Pajukanta: Locus for quantitative HDL-cholesterol on chromosome 10q in Finnish families with dyslipidemia. *Journal of Lipid Research* 2004, 45:1876-1884.

IV Päivi Pajukanta, Heidi E. Lilja, Janet S. Sinsheimer, Rita M. Cantor, Aldons J. Lusis, Massimiliano Gentile, Xiaoqun Joyce Duan, Aino Soro-Paavonen, Jussi Naukkarinen, Janna Saarela, Markku Laakso, Christian Ehnholm, Marja-Riitta Taskinen and Leena Peltonen: Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nature Genetics* 2004, 36:371-376.

*These authors contributed equally to this work
Publication II also appears in the thesis of Aino Soro-Paavonen (2004)

ABBREVIATIONS

ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
ApoA1	apolipoprotein AI
ApoA1C3A4	apolipoprotein AI-CIII-AIV
ApoA2	apolipoprotein AII
ApoA4	apolipoprotein AIV
ApoA5	apolipoprotein AV
ApoB	apolipoprotein B
ApoC2	apolipoprotein CII
ApoC3	apolipoprotein CIII
ApoE	apolipoprotein E
ASP	affected sib-pair
bp	base pair
CE	cholesterol esters
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
cM	CentiMorgan
DM	diabetes mellitus
DNA	deoxyribonucleic acid
FCHL	familial combined hyperlipidemia
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
HL	hepatic lipase
HMG-Co-A	3-Hydroxy-3-methylglutaryl coenzyme A
HRR	haplotype relative risk
IBD	identical by descent
IBS	identical by state
IDL	intermediate density lipoprotein
kb	kilobase
LCAT	lecithin:cholesterol acyltransferase
LD	linkage disequilibrium
LDL	low density lipoprotein
LPL	lipoprotein lipase
LOD	logarithm of odds
PAF-AH	platelet-activating factor acetylhydrolase
PCR	polymerase chain reaction
PL	phospholipids
PLTP	phospholipid transfer protein
PON1	paraoxonase 1
QTL	quantitative trait locus
SNP	single nucleotide polymorphism
SR-BI	scavenger receptor, class B type I
TC	total cholesterol
TDT	transmission/disequilibrium test
TG	triglyceride
type 2 DM	type 2 diabetes mellitus
VLDL	very low density lipoprotein

ABSTRACT

Familial combined hyperlipidemia (FCHL) and serum low HDL-cholesterol (HDL-C) are both highly atherogenic, complex lipid disorders, forming important risk factors for coronary heart disease (CHD), the leading cause of death in Western societies.

FCHL is the most common familial dyslipidemia predisposing to coronary heart disease (CHD) and estimated to be present in about 20% of premature CHD patients. FCHL is characterized by elevated serum total cholesterol (TC), triglycerides (TG), or both, and has many phenotypic features overlapping with the metabolic syndrome and type 2 diabetes mellitus (DM). Low HDL-C is a well-known component trait of both FCHL and type 2 DM. Actually low HDL-C, whether combined with other lipid abnormalities or not, is the most frequent dyslipoproteinemia in patients with premature myocardial infarction. Sharing the phenotypic similarities, these two CHD risk factors are likely to have a close pathophysiologic relationship as well as at least a partially overlapping genetic background.

To analyze the genetic factors underlying low HDL-C and FCHL, we studied multigenerational Finnish families with these lipid disorders and, consequently, premature CHD. We employed several approaches in our analyses. First, we studied candidate genes known to be involved in HDL-C metabolism in 25 low HDL-C families. Among 21 potential candidate genes relevant to lipid metabolism, we found suggestive evidence for linkage with two loci located in the genomic regions of the apolipoprotein A2 (APOA2) gene and the apolipoprotein A1C3A4 (APOA1C3A4) gene cluster.

Second, we performed a genome-wide scan to identify new loci and finally novel genes regulating the metabolic pathways determining serum HDL-C levels. A pooled data analysis of low HDL-C and FCHL study samples provided linkage to 8q23, 16q24.1-24.2 and 20q13.11 with low HDL-C trait. The strongest statistical evidence for linkage was observed on chromosome 8q23 (a lod score of 3.9). Importantly, this locus has earlier been linked to HDL-C levels in Mexican Americans. Further, the region on chromosome 20q has previously been linked to body adiposity, hyperinsulinemia and type 2 DM, suggesting that these traits and HDL-C have a partially shared genetic background.

Next, to further investigate the importance of 8q23, 16q24.1-24.2, and 20q13.11, we analyzed these three low HDL-C loci, as well as two loci for premature CHD on 2q31 and Xq24, and a locus for serum TGs on 10q11, in an extended study material. In this combined study sample of 92 low HDL-C and FCHL families, we fine mapped these six loci and tested their potential involvement especially in quantitative lipid traits. The quantitative HDL-C trait provided strong evidence for linkage to 10q11 (a lod score of 3.3). Furthermore, this region showed evidence of association for a quantitative trait obtained by combining quantitative HDL-C and TGs (p-value 0.0006). The data suggest that the locus on chromosome 10q11, previously linked to TGs, BMI and obesity, influences variation in plasma HDL-C and TG levels in Finnish dyslipidemic families.

The first major locus for FCHL on chromosome 1q21–q23 was identified in a genome-wide scan of Finnish FCHL families in 1998. Since then, this region has been replicated

in FCHL families originating from other populations. Interestingly, the same region has also been linked to type 2 DM in multiple studies. Since FCHL and type 2 DM share several phenotypic features, these data suggest that the same gene may underlie the obtained linkage results. To identify the FCHL gene on 1q21, we sequenced four functionally relevant regional candidate genes, human thioredoxin interacting protein (TXNIP), upstream transcription factor 1 (USF1), retinoid X receptor gamma (RGRG), and APOA2. We also genotyped 60 single nucleotide polymorphisms (SNPs) for 26 genes residing in the linked region. In these analyses, significant evidence for association, linkage and shared haplotypes was found between USF1 and FCHL ($p=0.00002$), especially in males with high TGs ($p=0.0000009$). Furthermore, expression profiles of fat biopsies of FCHL cases differed depending on their carrier status for the associated USF1 haplotype.

In conclusion, USF1 seems to confer susceptibility to high serum lipid levels. Although additional studies are warranted to address the functional differences between different USF1 alleles and their relevance for the FCHL phenotype, our results open novel insights into the genetic background of FCHL, the most common familial lipid disorder predisposing to CHD.

INTRODUCTION

Low serum HDL-C and FCHL represent important risk factors for atherosclerotic vascular disease, and are estimated to explain about 50% of familial dyslipidemias predisposing to premature CHD. Both are typical complex disorders, influenced by several environmental and genetic factors. FCHL is characterized by elevated levels of serum TC and TGs, and also expresses low HDL-C as a component trait. Low HDL-C and the lipid abnormalities of FCHL also overlap with the phenotypic features observed in the metabolic syndrome, a condition characterized by a clustering of risk factors for CHD. The metabolic syndrome significantly increases the risk of death from cardiovascular causes.

When searching for susceptibility genes underlying multifactorial disorders such as low HDL-C and FCHL, the relatively isolated Finnish population is likely to offer some advantages. The Finnish gene pool owes its special characteristics to its relative isolation and unique history of inhabitation involving multiple genetic bottlenecks, which have resulted in a founder effect and increased genetic drift (Norio 2003a; Norio 2003b). Thus, it can be assumed that, in this population, fewer predisposing genes and alleles underlie even complex diseases than in more mixed populations.

The Human Genome Project was completed in April 2003. Human genome sequence data accelerate the search for genes involved in complex traits. The new data freely available for everyone in World Wide Web have also been invaluable for this study by enabling utilization of new methods and strategies in dissection of the genetic background of common dyslipidemias.

The main purpose of this study was to investigate the genetic factors determining low HDL-C and FCHL in multigenerational Finnish families with these lipid disorders and premature CHD. We detected several loci for these lipid abnormalities, and finally identified a novel susceptibility gene for FCHL. This gene, USF1, located on 1q21, also offers an interesting candidate for the metabolic syndrome and type 2 DM which have been linked to this chromosomal region in several previous studies.

REVIEW OF THE LITERATURE

1. OVERVIEW OF LIPOPROTEIN METABOLISM

Plasma lipids are transported as water-soluble lipoproteins in the circulation. Lipoproteins consist of a hydrophobic core of cholesterol esters (CE) and TGs, surrounded by surface of more polar components of apolipoproteins, free cholesterol and phospholipids (PL). Plasma lipoproteins are separated into five major classes by ultracentrifugation according to their densities (Table 1) (Gotto et al. 1986).

Table 1. The principal lipoproteins (modified from Ganong 1997).

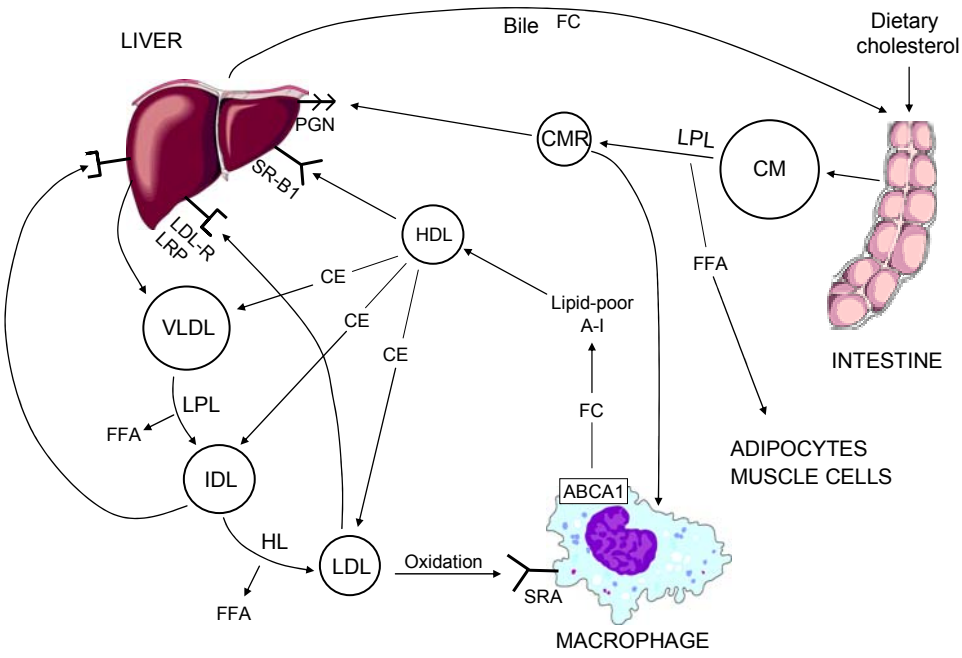
LIPOPROTEIN	ABBR	DENSITY (g/ml)	COMPOSITION %					PRIMARY APOs	ORIGIN
			Prot	FC	CE	TG	PL		
Chylomicrons	Cm	<0.93	2	2	3	90	3	apoB48, apoC-II	Intestine
Very low density lipoproteins	VLDL	0.94-1.006	8	4	16	55	17	apoB100, apoC-II	Liver, intestine
Intermediate density lipoproteins	IDL	1.007-1.019	10	5	25	40	20	apoB100, apoE	VLDL
Low density lipoproteins	LDL	1.019-1.063	20	7	46	6	21	apoB100	IDL
High density lipoproteins	HDL	1.063-1.210	50	4	16	5	25	apoA-I, apoA-II	Liver, intestine

APOs indicates apolipoproteins; CE, cholesterol esters; FC, free cholesterol; PL, phospholipids; Prot, proteins; TG, triglycerides.

An overview of lipoprotein metabolism is shown in Figure 1. The largest lipoprotein particles, chylomicrons (Cm), are formed in the mucosal cells of the intestine during the absorption of digested exogenous (dietary) lipids and secreted bile in the gut. Cm are cleared from the circulation by the action of lipoprotein lipase (LPL). This enzyme, located on the surface of the endothelium of the capillaries, catalyzes the breakdown of the TGs in the Cm to free fatty acids (FFA) and glycerol, and thus modifies Cm to chylomicron remnants. These cholesterol-rich Cm remnants are taken up by the liver. Delayed remnant removal from plasma by the liver has been suggested to be associated with premature atherosclerosis (Mamo et al. 1998).

Cm provide peripheral tissues with an important source of energy through the ß-oxidation of fatty acids. The excess energy of TGs is stored in adipose cells through the reesterification of FFA. During fasting, a portion of these TGs, derived from adipose tissue, re-enter the circulation as FFA and are transported to the liver where they can be used for energy or re-packed into very low density lipoproteins (VLDL). VLDL particles are formed in the liver and contain primarily TGs and CE. These particles are secreted into the circulation where they are then modified by LPL into intermediate density lipoproteins (IDL). IDL can acquire CE from HDL through the mutual exchange of TG by the action of the plasma enzyme cholesterol ester transfer protein (CETP). Some IDL are taken up by the liver and the remaining IDL are further modified into low density lipoproteins (LDL) after losing more TGs by the hydrolytic activity of LPL and hepatic lipase (HL) (Demant et al. 1988).

Figure 1. Overview of the lipoprotein metabolism.



ABCA1 indicates adenosine triphosphate-binding cassette transporter 1; A-I, apolipoprotein A-I; CE, cholesterol esters; CM, chylomicrons; CMR, chylomicron remnants; FC, free cholesterol; FFA, free fatty acids; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LRP, low-density lipoprotein receptor-related protein; LPL, lipoprotein lipase; PGN, proteoglycans; PL, phospholipids; SRA, scavenger receptor; SR-B1, scavenger receptor B1; VLDL, very-low-density lipoprotein.

LDL provide cholesterol to the tissues via LDL-receptor mediated uptake, where apoB100 acts as a ligand. Oxidized LDL is also taken up by the scavenger receptor of the macrophages (Brown et al. 1980). Macrophages overloaded with oxidized LDL are called foam cells, a cellular component of atheromatous plaque.

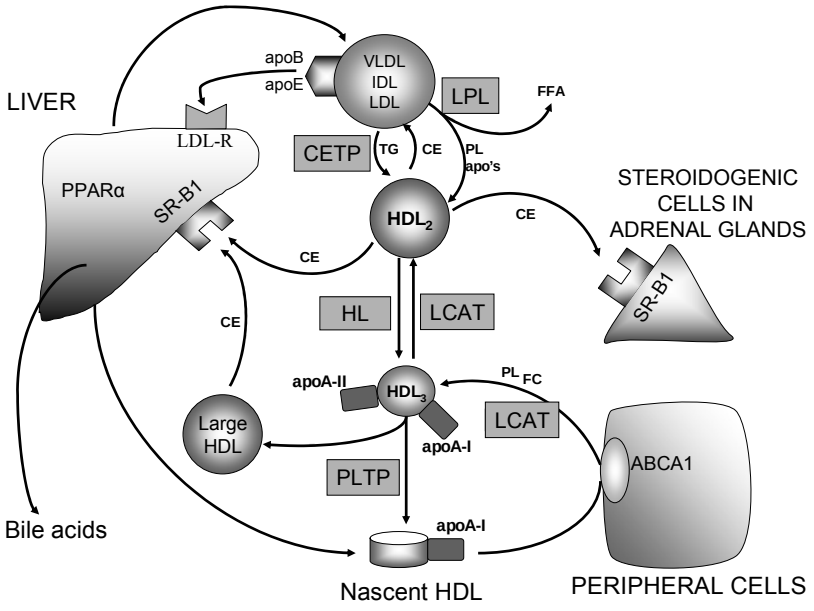
The small intestine and liver additionally synthesize nascent HDL, which returns excess cholesterol from peripheral tissues to the liver (reverse cholesterol transport) for excretion from the body in the bile (Rader 2003). In reverse cholesterol transport, HDL absorb cholesterol from peripheral tissues and importantly, from macrophages/foam cells (Chinetti et al. 2000; Brewer and Santamarina-Fojo 2003). CE of HDL particles are transferred through CETP to TG-rich lipoproteins, namely to IDL and LDL, and, eventually, back to the liver. The increased transfer of CE from HDL to IDL, and from LDL to VLDL, in return for TGs, contribute to the formation of highly atherogenic, small-dense LDL particles (Austin et al. 1990b; Krauss 1994; Packard et al. 2000; Kwiterovich 2002; Deeb et al. 2003).

1.1 HDL

High density lipoproteins, the smallest particles of the five lipoprotein classes present in the circulation, are a heterogeneous population of discoidal and spherical particles of differing size and composition. HDL particles are divided into two major subclasses by ultracentrifugation, HDL3 (d=1.125-1.250 g/ml) and HDL2 (d=1.063-1.0125 g/ml). HDL2 particles are about 50% larger and contain more cholesterol and TGs than HDL3 particles (Eisenberg 1984; Gotto et al. 1986).

Two major protein components of HDL-C particles are apolipoprotein-AI (apoA-I) (70%) and apolipoprotein-AII (apoA-II) (20%). ApoA-I is found in all HDL subclasses, whereas several other smaller apolipoproteins, apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, apoIV, apoD, apoE, and apoJ, are found in mature HDLs (Barter et al. 2003). Additionally, lipid transfer proteins (phospholipid transfer protein, PLTP; CETP), and modifying enzymes (lecithin:cholesterol acyltransferase, LCAT; paraoxonase 1, PON1; platelet-activating factor-acetylhydrolase, PAF-AH) exist in HDL. HDL particles are constantly remodeled by interaction with lipases, lipid transfer proteins, and cell-surface HDL receptors (Figure 2).

Figure 2.
Overview of the metabolic pathways of HDL and reverse cholesterol transport. Dietary cholesterol is not shown. TG indicates triglycerides, CE cholesterol esters, PL phospholipids and FFA free fatty acids.



Many proteins are known to be involved in HDL metabolism and to have a role in the regulation of HDL-C levels (Figure 2). These proteins can be divided into five groups: (1) HDL related apolipoproteins, (2) HDL associated enzymes and transfer proteins, (3)

plasma and cell enzymes that affect HDL, (4) cellular receptors and transporters that interact with HDL, and (5) transcription factors that influence HDL (Wang and Paigen 2002). Multiple mechanisms contribute to the transfer of cellular cholesterol to HDL. These include the ABCA1 transporter, which catalyzes active transfer of cholesterol from the cholesterol-rich cell surface to the HDL particle, and passive (nonspecific) transfer of cholesterol from the cell membrane via intermediates including serum albumin.

2. CLINICAL AND METABOLIC CHARACTERISTICS OF FAMILIAL LOW HDL-C AND FCHL DISORDERS

2.1 Low HDL-cholesterol

A decreased HDL-C level (also called hypoalphalipoproteinemia) is the most common lipoprotein abnormality in patients with premature coronary artery disease (Genest et al. 1992). High levels of HDL-C protect against CHD (Castelli et al. 1977; Gordon et al. 1977; Wilson et al. 1988; Gordon et al. 1989), and low HDL-C is a highly atherogenic disorder. Familial low HDL-C is a complex disorder, where genetic factors have a key role in regulating HDL-C levels (Prenger et al. 1992; Knoblauch et al. 1997). Rare forms of genetic HDL-C deficiency have been described, but the major loci affecting plasma HDL-C levels are still unknown. It has been estimated, that 40 to 60% of the variation in HDL-C levels are caused by genes (Hamsten et al. 1986; Prenger et al. 1992; Bu et al. 1994; Cohen et al. 1994; Guerra et al. 1997; Knoblauch et al. 1997; Inazu et al. 2001).

While genetic factors are major determinants of HDL-C (Hamsten et al. 1986; Cohen et al. 1994), environmental factors also influence HDL-C levels. Environmental factors include unmodifiable contributors such as age and gender. Modifiable lifestyle factors include alcohol consumption (Ellison et al. 2004), physical activity (Durstine et al. 2001), and smoking (Garrison et al. 1978; Criqui et al. 1980). In addition, BMI and TG levels are important determinants of HDL (Anzalone et al. 1995; Devroey et al. 2004), and low HDL-C is common in patients with abdominal obesity, type 2 DM, and the metabolic syndrome.

Decreased HDL-C (<35mg/dl or 0.9 mmol/L) is an independent risk factor for CHD (Schaefer et al. 1994). Therefore, HDL metabolism is an attractive target for drug development in order to elevate HDL levels and decrease the risk for CHD. Currently there are no pharmacological interventions available that specifically raise HDL-C and leave other lipid levels unchanged. However, complex HDL metabolism offers potential opportunities for drug discovery. For example, pharmaceutical approaches to HDL elevation include niacin and the fibric acid derivatives, which both raise HDL-C and have been shown to decrease the risk for CHD (Safeer and Cornell 2000).

The role of HDL as an independent inverse predictor of CHD has been firmly established by numerous epidemiologic studies (Castelli et al. 1986; Assmann et al. 1996). HDL is involved in reverse cholesterol transport, where HDL particles deliver excess cholesterol from the peripheral tissues to the liver or transfer cholesterol into VLDL in a process mediated by CETP (Figure 2). In addition to reverse cholesterol transport, HDL has many other antiatherogenic roles (Table 2) (reviewed in Navab et al. 2001; von Eckardstein et

al. 2001; Assmann and Nofer 2003; Assmann and Gotto 2004). For instance, HDL acts as an anti-inflammatory and antioxidant agent (Table 2).

Table 2.

ANTIATHEROGENIC ROLES OF HDL
Reverse cholesterol transport
Anti-thrombotic properties
Pro-fibrinolytic activity
Antioxidant effect: Prevention of LDL oxidation
Anti-inflammatory effect: Inhibition of endothelial adhesion molecule expression

Interestingly, inflammatory mechanisms are increasingly recognized to play an important role in vascular disease, as inflammatory markers correlate with prognosis in acute and chronic CHD. Recently, HDL was shown to prevent the CRP-induced upregulation of inflammatory adhesion molecules (Wadham et al. 2004). It has been suggested that the antioxidant properties of HDL are carried out by the enzymes associated with this lipoprotein, such as PAF-AH, PON1, and LCAT (reviewed in Navab et al. 2001; Kovanen and Pentikainen 2003).

2.2 Familial combined hyperlipidemia

FCHL, first described in 1973 (Goldstein et al. 1973; Nikkila and Aro 1973; Rose et al. 1973), is one of the most common genetic dyslipidemias. About 10-20% of patients with premature CHD have this lipid disorder (Genest et al. 1992), and the population prevalence of FCHL is approximately 1-2% (Goldstein et al. 1973). In FCHL patients, serum TC, TGs or both are elevated (Goldstein et al. 1973; Nikkila and Aro 1973). FCHL is a common, complex disorder, where several genes, environmental factors and their interactions are involved. The heritability of TC and TGs has been estimated: up to 60% of the variability of these lipid levels is determined by genetic factors (Bucher et al. 1988).

In addition to hypercholesterolemia and hypertriglyceridemia, there are several other important component traits in FCHL, such as other atherogenic lipid abnormalities and features of insulin resistance syndrome. The list of these component traits is shown in Table 3.

Table 3. Component traits in FCHL.

ApoB containing lipoproteins ↑	Sniderman et al. 1980; Brunzell et al. 1983;
Small dense LDL particles ↑	Austin et al. 1990a; Hokanson et al. 1993
Glucose tolerance ↓	Hunt et al. 1989; Aitman et al. 1997;
Abdominal obesity ↑	Bredie et al. 1997a; Vakkilainen et al. 1998;
Insulin ↑	Pihlajamaki et al. 2000
HDL-C ↓	de Graaf and Stalenhoef 1998
Free fatty acids ↑	Castro Cabezas et al. 1993
LPL activity ↓	Babirak et al. 1992

The FCHL phenotype overlaps with several common disorders, such as hyperapobetalipoproteinemia, LDL subclass pattern B (small, dense LDL particles), and most interestingly, the metabolic syndrome and type 2 DM (earlier known as non-insulin-dependent diabetes mellitus, NIDDM) (Ayyobi and Brunzell 2003). Importantly, a potential genetic overlap between FCHL, the metabolic syndrome, and type 2 DM has been suggested (Lewis 2002; Ayyobi and Brunzell 2003), making studies of the genetic background of FCHL relevant for these diseases as well.

2.3 The metabolic syndrome and type 2 DM

The metabolic syndrome is a condition characterized by a clustering of CHD risk factors such as insulin resistance, elevated fasting plasma glucose, abdominal obesity, elevated blood pressure, and atherogenic dyslipidemia, including elevated triglycerides and decreased HDL-C level (Grundy 1999). The National Cholesterol Education Program (NCEP) and the World Health Organization (WHO) recently published definitions for the metabolic syndrome. The criteria for the clinical diagnosis for the metabolic syndrome by the Adult Treatment Panel III (ATPIII) are shown in Table 4.

Table 4. The criteria for the clinical diagnosis for the metabolic syndrome by the Adult Treatment Panel III (ATPIII) (NCEP 2001).

Waist circumference	> 102 cm ♂, >88 cm ♀
TGs	>1.7 mmol/l
HDL-C	<1.03 mmol/l ♂, <1.29 mmol/l ♀
Blood pressure	≥ 130/85 mmHg
Fasting glucose	≥ 6.1 mmol/l

At least three of these criteria have to be fulfilled to diagnose the metabolic syndrome. According to these criteria, the prevalence of the metabolic syndrome is over 20% in US (Park et al. 2003) and about 10 % in Finland (Lakka et al. 2002).

The WHO has also published definitions for the metabolic syndrome. According to the WHO, the metabolic syndrome is defined as insulin resistance or the presence of impaired glucose tolerance or type 2 DM and the presence of at least two of the following: abdominal obesity (waist-hip ratio > 0.90 in males and > 0.85 in females or BMI 30 kg/m2), dyslipidemia (serum TGs ≥ 1.70 mmol/l or HDL-C < 0.9 mmol/l in males and <1.0 mmol/l in females), hypertension (140/90 mmHg), or microalbuminuria (Alberti and Zimmet 1998; WHO 1999).

It has been suggested that in type 2 DM patients, the ATPIII proposal more clearly identifies the burden of CHD associated with the metabolic syndrome as compared to the WHO criteria (Marchesini et al. 2004). The WHO definition, however, seems to have relatively high sensitivity and specificity in predicting diabetes (Laaksonen et al. 2002).

The metabolic syndrome is a proinflammatory- and prothrombotic state, where cardiovascular disease is a major clinical outcome. Two subgroups of patients with the metabolic syndrome are suggested to have a particularly high risk for premature CHD: individuals with type 2 DM or FCHL (Carr and Brunzell 2004). These traits account for

up to 50% of early cardiovascular disease risk in patients with the metabolic syndrome. Furthermore, the metabolic syndrome is a very common disorder. For example, it has been estimated to affect 47 million Americans (NCEP 2001). The metabolic syndrome is also closely related to type 2 DM, which is a serious health problem, reaching epidemic proportions worldwide, especially in developed countries.

Dyslipidemia is a typical feature of type 2 DM (Kannel and McGee 1979), and partly because of the atherogenic lipid profile, CHD is the leading cause of death in type 2 DM (Garcia et al. 1974). The most common lipid abnormalities present in type 2 DM are elevated TGs and decreased HDL-C levels (Howard 1987). The lipid abnormalities present in type 2 DM are the same as in the metabolic syndrome, but the dyslipidemic phenotype in type 2 DM tends to be more severe (Taskinen 2003). In addition to dyslipidemia, type 2 DM is also characterized by abnormalities in carbohydrate metabolism (DeFronzo 1997).

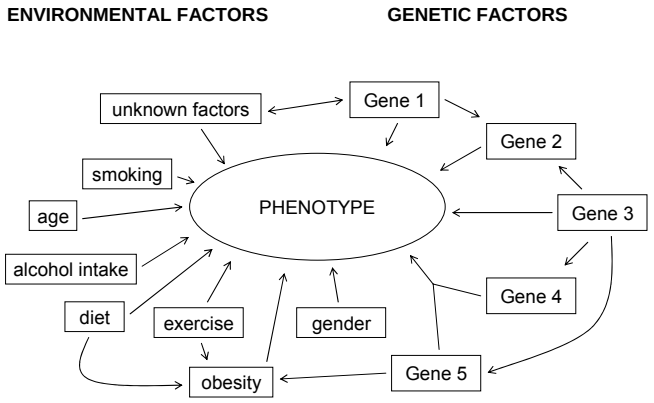
As in FCHL and in the metabolic syndrome, the causes of type 2 DM are also multifactorial, including both genetic and environmental elements (Froguel and Velho 2001). The typical features in the pathogenesis of type 2 DM are reduced beta-cell dysfunction and insulin sensitivity, which, together with an obesity-promoting lifestyle and genetic susceptibility alleles, predispose to the disease.

The incidence and prevalence of type 2 DM is increasing almost epidemically due to ageing of the population structures in developed countries, globally increasing obesity and sedentary lifestyles. It has been estimated that, by the year 2010 the total number of people with diabetes will reach 220 million (Amos et al. 1997). Moreover, the worldwide number of cases with diabetes is predicted to double by the year 2025 from a currently estimated 150 million to 300 million (King et al. 1998; WHO 2003). The greatest number of cases is expected in developing countries such as China and India (King et al. 1998).

3. COMPLEX TRAITS AND THE ISOLATED POPULATION OF FINLAND

Complex traits such as FCHL, CHD, DM, and hypertension are consequences of multiple genetic and environmental factors, and their interactions (Figure 3). Such multifactorial genetic disorders, caused by the interactions of multiple genes and the environment, do not follow the classical Mendelian inheritance patterns and are thus called complex traits. Complicated interactions between modifier and susceptibility genes modulate the phenotype of individuals with diseases. For instance, protective alleles can suppress disease in otherwise susceptible individuals. The modifier genes modulate penetrance, dominance, pleiotropy or expressivity in individuals with Mendelian traits and most likely affect complex traits as well (Nadeau 2003).

Figure 3.
CHD and FCHL are typical complex traits where several susceptibility and modifying genes, environmental factors, and their complicated interactions underlie the disease phenotype.



In multifactorial diseases, several typical confounding factors (Table 5) make the investigation of the genetic background exceptionally challenging (Lander and Botstein 1986; Lander and Schork 1994; Risch 2000). For example, complex traits may result more often from noncoding regulative variants than from coding sequence variants (Mackay 2001; Korstanje and Paigen 2002). Thus, to find causal nucleotide variants underlying complex traits, sequencing of coding regions is not enough. Also interpreting the consequences of noncoding sequence variants is complicated, because the relationship between a promoter or intergenic sequence variation, gene expression level, and a trait phenotype is not as well understood as the relationship between the coding variants and protein function (Glazier et al. 2002).

Table 5.

FACTORS COMPLICATING GENETIC ANALYSES OF COMPLEX TRAITS
Difficulties in diagnosis and classification of the phenotype
Variable expressivity of the phenotype
Late onset of the disease
Quantitative phenotypes
Unknown mode of inheritance
Phenocopies
Unknown or low penetrance
Pleiotrophy
Epistasis
Common disease-predisposing alleles
Genetic heterogeneity
Limited statistical power
Multiple testing
Publication bias

Some efforts can be made to reduce the impact of confounding factors in the background of common complex diseases. For instance, focusing on familial cases with well-defined clinical phenotypes, early onset of the disease and severe phenotype, and exclusion of obvious environmental risk factors should enrich the genetic forms of the disease. Concentration on families originating from population isolates as well as investigation of animal models can further help tackle the genetic background of complex diseases and may help dissect the molecular mechanisms underlying these disorders.

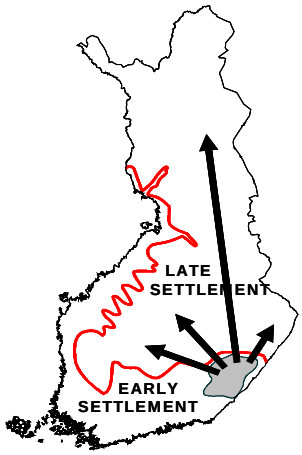
Genetic and environmental heterogeneity are reduced in genetically isolated populations such as Finland (Peltonen et al. 1995), Sardinia (Angius et al. 2001), Costa Rica (Mathews et al. 2004), the Canadian Hutterites (Ober et al. 2001), the Old Order Amish (Cross 1976), the North American Mennonites (Jaworski et al. 1989), the Ashkenazi Jews (Permutt et al. 2001), and the Benkala in Bali (Friedman et al. 1995). When studying individuals originating from population isolates, the impact of genetic involvement is more homogeneous than in mixed populations. Thus, possibilities of identifying contributing genes for common lipid disorders such as FCHL are likely to be increased. It has been suggested that stabile populations such as the Saami should be well suited for mapping complex traits, whereas rapidly expanded populations such as the Finns for mapping monogenic diseases (Laan and Paabo 1997; Terwilliger et al. 1998).

Although there have been some inhabitants in Finland since the end of glacial period about 11 000 years ago (Nevanlinna 1972; de la Chapelle 1993), Finland has mainly been inhabited in two waves. Early settlement occurred more than 2000 years ago and populated mainly the coast and the southern part of Finland (Nevanlinna 1972; de la Chapelle 1993). Late settlement took place during the 16th century, from a small south-eastern area to the central, western and finally northern parts of the country, thus inhabiting wilderness and yet non-inhabited parts of Finland (Figure 4). This internal migration movement in the 1500s produced internal isolates and regional subisolates. Because of the persistence of the subisolates, the prevalence of some mutations differs regionally, and local clusterings of some disease alleles exist (Pastinen et al. 2001).

According to another inhabitation theory called the dual-origin hypothesis, two different groups settled Finland: one from east, and the other from south (Eriksson 1973; Norio et al. 1973). Genetic evidence from Y chromosome supports this dual-origin model (Kittles et al. 1998). Despite the opposing inhabitation theories it has been proposed that the current Finnish population is related to other European populations (Sajantila et Paabo1995; Lahermo et al. 1996). A recent review article by Norio (Norio 2003b) discuss about the settlement of Finland and summarizes that the Finns are about 10 000 year-old north-Europeans, who also have genes from southern Europe and additionally, some genes as a result of movement in east-west direction.

For geographical, linguistic and cultural reasons, the Finnish population remained isolated for about 2000 years. In addition, multiple bottlenecks (such as wars, diseases, famine years) molded the gene pool. The Finnish population has also grown exponentially from 18th century to date, from 250 000 inhabitants to 5.2 million.

Figure 4. The internal migration movement of the 16th century. (Modified from Peltonen et al. 1999)



The relatively isolated population of Finland has been shown to offer many advantages for genetic research and studies of monogenic traits (Peltonen et al. 1999), and these benefits might also help to dissect complex disorders (Peltonen et al. 2000). In addition to isolation, the founder effect and genetic drift have also had an impact on the population. In the founder effect, a new colony is formed by a very small number of individuals from a larger population and this produces a loss of genetic variation. In genetic drift, changes in the frequencies of alleles in a population occur by chance, rather than because of natural selection. The magnitude of the gene frequency changes due to genetic drift is inversely related to the size of the population; the larger the number of reproducing individuals, the smaller the effects of genetic drift.

Why do harmful alleles exist in a population? Gene selection in the past has probably been influenced by conditions of life in those days, for example famine (Damcott et al. 2003; Chakravarthy and Booth 2004). To ensure survival during periods of famine, some alleles that may have been advantageous in ancient times may have concentrated in population. This heterozygote advantage means that the high rate of occurrence of a condition that is usually very harmful or even lethal in its homozygous form may allow greater survival for heterozygotes for example during famine. Thus heterozygotic carriers of the gene are more likely to survive to adulthood and therefore to pass their genes to the next generation.

The alleles that used to be advantageous in the distant past may today be disadvantageous and cause for instance obesity and predispose to CHD because of the lifestyle of the modern-day humans including physical inactivity and a positive caloric balance. The genes that constitute a risk for developing obesity-related conditions, such as type 2 DM and the metabolic syndrome, when energy is abundant and that on the other hand, conserve energy during famines are called thrifty genes. “Thrifty genotype”, defined as being exceptionally efficient in the intake and/or utilization of food, was initially proposed by Neel (Neel 1962), who argued that certain genotypes were selected into the

human genome because of their selective advantage over the less "thrifty" ones. The FOXC2 gene residing in the 16q region linked to low HDL-C (Pajukanta et al. 2003) has been proposed to work as an anti-thrifty gene in hypertriglyceridemia, obesity, and diet-induced insulin resistance (Cederberg et al. 2001). A high fat diet induces FOXC2 expression in adipocytes, leading to a lean and insulin sensitive phenotype. Thus, increased FOXC2 levels could be protective against type 2 DM (Cederberg et al. 2001).

Due to the founder effect and to isolation, monogenic diseases are less likely have both locus and allelic heterogeneity, and affected individuals tend to share ancestral haplotypes derived from a handful of founders. Thus, strategies using linkage disequilibrium (LD) and a shared haplotype in affected individuals can be applied to restrict the critical DNA region efficiently (Peltonen et al. 1999; Peltonen et al. 2000). In fact, LD has been successfully used in mapping of genes for numerous rare monogenic diseases which belong to the Finnish disease heritage (Peltonen et al. 1999). However, later on it has become evident that monogenic traits are often not as simple as was expected (Nabholz and von Overbeck 2004). Several different mutations in the same or in different loci, with variable phenotypic effects and highly variable associated risks have been identified underlying diseases of simple Mendelian inheritance. For example, the Marfan syndrome has turned out to be fairly heterogeneous, showing both locus and allelic heterogeneity. The Marfan syndrome can be caused by mutations of two different genes and, on the other hand, patients with the same mutation can show a wide degree of phenotypic variability (Collod-Beroud and Boileau 2002).

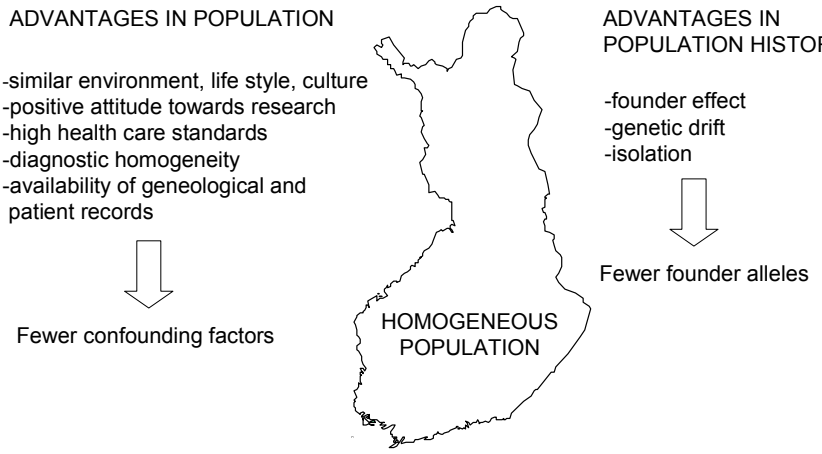
As in monogenic diseases, there might be fewer disease predisposing alleles also in multifactorial diseases in the relatively isolated population of Finland, compared to populations of more heterogeneous origin (Lander and Botstein 1986; Lander and Schork 1994; Collins 1995; Peltonen et al. 2000). Accordingly, Finnish study samples, consisting of many large pedigrees with multiple affected individuals, have been used in genetic studies of multiple complex diseases (Table 6). These studies have in most cases resulted in the initial positioning of several susceptibility loci, and many of these loci have also been detected in other populations. For example, the first major locus for FCHL in 1q21-23 was detected in Finnish FCHL families (Pajukanta et al. 1998). Since then, this linkage has also been replicated in other, more heterogeneous study samples (Coon et al. 2000; Pei et al. 2000; Allayee et al. 2002).

Table 6. Examples of genome scans for common traits performed previously utilizing Finnish study samples in the National Public Health Institute, Helsinki, Finland

TRAIT	AUTHORS AND YEAR
Multiple sclerosis	Kuokkanen et al. 1997
Schizophrenia	Ekelund et al. 2000; Paunio et al. 2001
Bipolar disorder	Ekholm et al. 2003
FCHL	Pajukanta et al. 1999
Low HDL-C	Soro et al. 2002
Premature CHD	Pajukanta et al. 2000
Hypertension	Perola et al. 2000
Osteoarthritis	Leppavuori et al. 1999
Obesity	Ohman et al. 2000
Autism and Asperger syndrome	Auranen et al. 2002; Ylisaukko-oja et al. 2004

Importantly, in population isolates like the Finns, a high degree of environmental homogeneity is also typical (Figure 5) (Peltonen et al. 2000). For example, life style, diet and culture tend to be more similar among isolated than in more mixed populations. In general, Finns also seems to have a positive attitude towards medical research. In addition, uniformity in physician training and clinical practice yields consistent diagnoses and phenotyping of diseases.

Figure 5. Advantages for genetic research in Finland: Reduced genetic and environmental heterogeneity.



4. STRATEGIES FOR MAPPING GENES UNDERLYING COMPLEX TRAITS

Since complex diseases do not follow classic and simple Mendelian inheritance patterns, the genetic dissection of complex traits is very challenging. Glazier et al. suggested a four-step strategy for gene discovery in studies of complex traits (Figure 6) (Glazier et al. 2002).

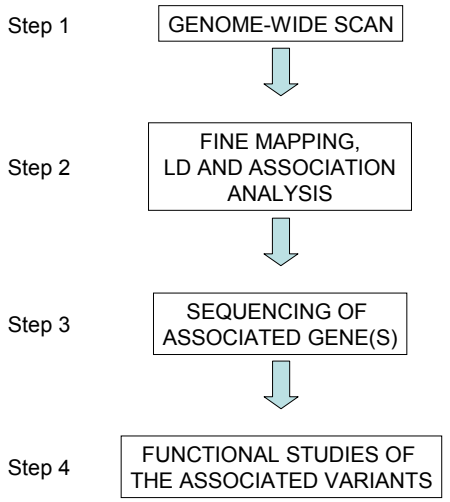
In step 1, a whole-genome linkage study is performed. Statistically significant evidence of linkage has to be established in a single study or consistent suggestive evidence has to be found in several independent studies (Lander and Kruglyak 1995; Risch and Merikangas 1996; Dahlman et al. 2002). Because of the nature of the complex traits, the minimal interval for the restricted locus in primary genome screens is usually no less than 10 to 30 cM (containing ~ 100 to 300 genes), a resolution that is sufficiently precise to justify further study (Glazier et al. 2002).

In step 2, the aim is to reduce the size of the critical region. This can be done in humans by utilizing genetic methods such as LD mapping, family-based studies, and case-control studies. These approaches may even reduce the minimal interval to 1 cM.

Identification of the candidate nucleotide variants residing in the region is performed in step 3. Nowadays this can be mainly done by utilizing SNP databases, and burdensome large-scale DNA sequencing analysis of the region is not needed any more. After identification of the SNPs, the relevance of each candidate nucleotide variant and haplotype has to be tested.

In step 4, to get conclusive evidence, associated candidate genes are functionally tested. The gene plays an important role in the trait, if replacement of the variant nucleotide results in changes in phenotype. This proves that a molecular variant is functionally associated with differences in phenotype. Functional tests can be carried out by traditional cell biology methods, such as overexpression studies in transfected cell lines, or by transgenic and gene-targeting technologies. For cellular phenotypes even in vitro functional tests may be appropriate (Glazier et al. 2002).

Figure 6. Overview of the a four-step strategy for gene discovery in studies of complex traits (Glazier et al. 2002).



Although the four-step strategy mentioned above has been commonly utilized previously, the strategy may change in the future, after the International HapMap Project has produced a haplotype map of the human genome. The HapMap will describe the common patterns of human DNA sequence variation and this information might facilitate genome-wide association studies in the search for genes behind complex traits (see 3.2.5 Genome-wide association studies).

4.1 Human Genome Project

In 2001, as a result of international collaboration in the Human Genome Project (HGP), a draft sequence of the human genome became freely available (Lander et al. 2001). At the

same time, the sequence of the human genome was also published by the Celera company (Venter et al. 2001). Since HGP was completed in April 2003, all of the sequence data generated by the International Human Genome Sequencing Consortium has been swiftly deposited in public databases and made freely available to scientists around the world (http://www.ncbi.nlm.nih.gov/genome/ guide/human/).

Although HGP is essentially completed, the exact number of genes encoded by the genome is still unknown. The latest estimates from gene-prediction programs suggest that there might be about 24 500 or fewer protein-coding genes (Pennisi 2003), much fewer than the previous estimates of around 100 000. This smaller number of human genes has been a surprise, because with around 30 000 genes, the human gene count is only about 30% greater than that of the simple roundworm C. elegans with about 20 000 genes (Table 7) (Claverie 2001), and counting genes has been viewed as a way of quantifying genetic complexity. However, people and chimpanzees have roughly 98 percent of their DNA in common; suggesting that the biological consequences due to the genetic differences are much more complicated than was previously speculated (Weissenbach 2004).

Table 7. The comparative genome sizes of human and other organisms sequenced.

Organism	estimated size	estimated gene number
Homo sapiens (human)	3000 million bases	~30,000
Mus musculus (mouse)	3000 million bases	~30,000
Drosophila melanogaster (fruit fly)	180 million bases	13,600
Arabidopsis thaliana (plant)	125 million bases	25,500
Caenorhabditis elegans (roundworm)	97 million bases	19,100
Saccharomyces cerevisiae (yeast)	12 million bases	6300
Escherichia coli (bacteria)	4.7 million bases	3200

The smaller number of human genes than was expected is consistent with the theory that variations in gene regulation and the splicing of gene transcripts produce many distinct functions for one protein in different tissues. It also seems that mutations in the coding sequences of genes are responsible for only a delimited number of the differences in disease susceptibility between individuals. In fact, sequence variants (SNPs) that affect gene splicing and regulation must play an important role in determining disease susceptibility (Peltonen and McKusick 2001). As only a few of the millions of SNPs in the genome have such functional impacts, identifying these SNPs is important. Therefore, one of the goals of HGP is to create SNP maps of the human genome.

It has been estimated that a SNP occurs every 200 to 300 bases along the 3.2-billion-base human genome, making up about 90% of all human genetic variation, and resulting in an estimated 11-15 million SNPs (Kruglyak and Nickerson 2001; Salisbury et al. 2003). SNP maps may help to identify the multiple genes associated with complex diseases, in which a single altered gene may make only a small contribution to the disease and therefore associations are difficult to establish with conventional gene-hunting methods (Chakravarti 2001). However, to understand how SNPs and other genetic variations influence the phenotypes and expose the individuals to diseases will be the major challenge for biomedical researchers in the next decades.

4.1.1 Comparative genomic approach

High-throughput genome sequencing of HGP has created a new era for biomedical research, and now the sequencing of entire genomes (the genomes of more than 160 organisms have been sequenced to date) has set the scene for gene discovery. However, while HGP describes the nucleotide composition of nuclear DNA and the anatomy of the genome, it tells us nothing about the phenotypes encoded in the genotypes. Thus, HGP gives us only the object and the tools to further define the real molecular background of diseases.

The increasing availability of genomic sequences from different species enables comparative genomic approaches (Nobrega and Pennacchio 2003; Pennacchio 2003; Pennacchio and Rubin 2003b), which has proved to be a powerful way for the functional annotation of the human sequence. Comparative genomic strategy also forms a feasible tool in the analysis of cardiovascular-related genes (Cheng and Pennacchio 2003).

A basic hypothesis of comparative genomics is that evolutionarily conserved sequences are functionally important, and thus have remained unchanged through time (Nardone et al. 2004). Based on this hypothesis, focusing on sequences that are highly conserved between different species, for example between human and mouse, can offer powerful tools for the discovery of biologically active gene regulatory elements and facilitate the identification of genes behind complex traits. This strategy was utilized to identify additional functional elements in the vicinity of the APOA1C3A4 gene cluster and, as a result, led to the identification of a novel apolipoprotein member of this gene cluster, APOA5 (Pennacchio and Rubin 2001). The animal model for the trait can also offer potent tools for dissecting the trait through experimental crosses, followed by studies of genetic homology in humans. After the genes are identified in mouse models, their human orthologues can be predicted.

4.2 Qualitative and quantitative traits

Human diseases can represent either a qualitative or quantitative trait. The qualitative trait is a so-called on-off trait, which cannot be measured but rather the disease is either present or not (e.g. albinism). However, quantitative phenotypes often underlie these on-off traits, when they are based on a superimposed cut-off value of the quantitative trait (e.g. hypertension).

Multifactorial diseases and especially their trait components are often quantitative traits showing continued variation, such as blood pressure in hypertension, BMI in obesity, glucose tolerance in diabetes, and plasma lipid levels in lipid disorders. Such measurable characteristics often follow a normal distribution with mean and variance, forming a bell shaped curve**.** A certain correlation is usually seen in measured values: tendency of the measured values is more similar among relatives than among the general population. This correlation can be either positive or negative, reflecting both genetic and environmental influences.

The locus affecting complex disorders is called the quantitative trait locus (QTL). The QTL is a polymorphic locus which contains alleles that differentially affect the expression

of a continuously distributed phenotypic trait. Generally, it is a genetic marker described by statistical association with quantitative variation in the particular complex trait that is thought to be controlled by the cumulative action of alleles at multiple loci.

Heritability is the fraction of the total phenotypic variance of a quantitative trait caused by genes, estimating the contributions of the different alleles to the variability of a particular quantitative trait. Heritability of a quantitative trait can also be estimated in twin studies, but this parameter does not reveal how many genes are involved or how the different genes interact.

Complex traits such as multiple sclerosis, obesity and CHD can also be analyzed as qualitative traits. In that case, an underlying liability distribution is assumed and, if a threshold of liability is exceeded, an individual becomes affected (polygenic threshold trait). For example in obesity, BMI is either above 30 and a person is obese (affected), or BMI is below 30 and a person is not obese (unaffected). The qualitative trait method has been used successfully in studies of complex disorders, in which families have been identified through an affected individual, otherwise known as a proband. Additional family members are collected to build multigenerational families in which genetic linkage studies can be undertaken.

4.3 Statistical methods for mapping complex disease genes

Several different statistical approaches can be utilized to map the genes involved in the etiology of common complex diseases, including parametric linkage analysis, nonparametric allele-sharing based methods (affected-sib-pair and affected-pedigree-member methods), association analysis, linkage-disequilibrium-based analysis, and polygenic analysis of experimental crosses (Lander and Schork 1994). The choice of the analysis method depends on the study sample available, the trait investigated (e.g. qualitative or quantitative) and prior knowledge of genetic determinants (e.g. positional or functional candidates, model organisms).

4.3.1 Parametric linkage analysis

The basic approach to genetic mapping is linkage analysis, which has been traditionally and successfully applied to simple monogenic traits. It can also be utilized in a linkage analysis of extended families in multifactorial diseases (Curtis et al. 1995). Linkage analysis is a parametric method for testing genetic linkage. In this parametric approach, several parameters need to be estimated: mode of inheritance and frequency of the disease gene, as well as penetrance (meaning the probability of being affected, given the certain genotype). In complex traits, all these parameters are typically unknown, leading to a more or less “wrong” model and, thus, reducing the possibilities and power to detect true linkage signals (Clerget-Darpoux et al. 1986). These problems can be partially avoided in nonparametric methods such as allele sharing methods, in which no specified parameters of the disease locus characteristics are needed.

Linkage analysis determines whether the alleles at the disease locus and at a polymorphic marker locus co-segregate in a pedigree more often than they would if they were located physically far away from each other or even on different chromosomes. In linkage

analysis, it is determined if the recombination fraction (θ) between the two loci differs significantly from θ=0.5, which is expected for unlinked loci. The likelihood-based lod score method (Morton 1955) can be used to analyze linkage within families. This method determines a lod score (Z), which is a log of the odds that two gene loci are linked versus unlinked. In linkage analysis, the overall likelihood of the data for two alternative assumptions is calculated: first, that the two loci are linked with the given recombination fraction (θ), and second that they are not linked. The logarithm to the base 10 of the ratio of these two likelihoods is the lod score (Z) for the "logarithm of the odds". Z=log10 is the likelihood of data if loci are linked with a given θ.

$$Z(\theta)=\log_{10}\frac{L(\text{linkage})}{L(\text{no linkage})}$$

In the formula above, L is the likelihood function and θ is the recombination fraction. The most likely distance between two loci (e.g. a marker and a disease gene) is the recombination fraction at which the lod score peaks. A lod score of 3.0 corresponds to a p-value of <0.001 (Ott 1991), and is considered to be significant evidence for linkage, whereas a lod score of -2.0 indicates significant evidence for exclusion of t linkage when investigating a monogenic disease.

Software programs, such as the LINKAGE package (Lathrop and Lalouel 1984; Lathrop et al. 1984; Lathrop et al. 1986), are commonly used to test linkage in practice. The LINKAGE package includes a series of programs for maximum likelihood estimation of recombination rates, and calculation of two-point and multipoint lod scores, as well as analysis of genetic risks.

4.3.2 Non-parametric linkage analysis: Allele-sharing methods

Non-parametric methods, also called "model-free" methods, partially circumvent some problems associated with linkage analyses. The reason for this is that non-parametric methods are not dependent on the estimates needed in the "model-based" methods, i.e. mode of inheritance, penetrance and gene frequency. In non-parametric methods, all meioses are considered as independent and equally informative for the disease gene. Nonparametric linkage analyses are allele-sharing methods, including affected-sib-pair and affected-pedigree-member methods.

Allele sharing methods are based on allele sharing of affected individuals. Two sibs share 0, 1 or 2 copies of any locus with a 25%-50%-25% distribution expected under Mendelian segregation. Excess allele sharing of a disease-associated locus can be calculated by a chi2 test. Thus, when there is evidence for allele sharing, the affected relatives share the allele(s) more often than is expected by chance. Any two copies of the same allele are called identical by state (IBS). Only if the shared allele is known to be inherited from a common ancestor, is the allele called identical by descent (IBD). Sometimes IBD status cannot be determined unequivocally. IBD methods are more powerful and less prone to errors in allele frequencies than IBS methods, but, especially in late-onset diseases, the parents' samples are often unavailable, making it impossible to use the IBD methods.

Affected sibpair (ASP) analysis is one of the allele sharing methods. For example, the SIBPAIR program (Kuokkanen et al. 1996) of the ANALYZE package (Terwilliger and Goring 2000) employs this method, in which affected sibpairs are studied to seek distortion from expected allele sharing. Between affected sibpairs, sharing of marker alleles and of phenotypes is more likely when the marker is closely linked to the segregating variation that causes trait variation.

4.3.3 Variance component linkage analysis

It has been suggested that, for quantitative traits such as plasma HDL-C levels, utilizing the full quantitative information of the trait variation is more powerful than arbitrary dichotomizing of the trait into a binary phenotype. There are several methods developed for QTL linkage analysis, such as Haseman-Elston regression (Haseman and Elston 1972) and variance-component methods (Amos 1994; Almasy and Blangero 1998; Blangero et al. 2001). Currently, the variance-component method is one of the most frequently applied approaches in studies attempting to localize QTLs, since, in contrast to regression-based methods, the variance component model can be extended to incorporate covariates, gene-environment interactions and other confounding factors.

The variance-component linkage approach is based on specifying the expected genetic covariances between relative pairs as a function of the identity by descent (IBD) allele sharing relationships at a given QTL (Amos 1994; Almasy and Blangero 1998). Variance component analyses are optimal for large population study samples, where the families are not collected on the basis of some disease. When a sample is non-randomly selected through probands, it might inevitably result in a limited variation for the quantitative trait studied, for instance in serum lipid levels, and thus in an ineffective QTL analysis. Thus, ascertainment correction is generally required. This is done to obtain unbiased parameter estimates when applying variance component methods (Amos and de Andrade 2001).

The variance component approach can be used to analyze large pedigrees, and it is able to accommodate multiple loci simultaneously in a true oligogenic model. The genotype-environment interaction and epistasis can also be examined in a variance component framework. The SOLAR computer package is an example of variance component methods (Almasy and Blangero 1998) that implements general pedigree variance component and IBD estimation methods. SOLAR is able to include non-genetic factors (e.g., age, gender) as covariates. It can also be used to perform multipoint linkage analysis of multiple QTLs.

4.3.4 Association analysis

Association and LD analysis are usually used to analyze candidate genes or to refine the localization of the disease gene, in studies of both monogenic and multifactorial diseases. Association analysis compares the frequency of alleles of the locus between cases and controls. Parental alleles that are not transmitted to the patients can also be used as controls in family-based association analysis, and hence avoid the typical problem of matching control group and cases. The haplotype relative risk (HRR) approach (Falk and Rubinstein 1987; Terwilliger and Ott 1992) and transmission disequilibrium test (TDT) (Spielman et al. 1993) are such family-based methods. In the HRR test, homozygous

parental genotypes also provide some information for the analysis, whereas in the TDT approach they do not. If association is detected, it can mean (1) Direct cause of disease, i.e. the marker is causally implicated in the disease (2) Linkage disequilibrium i.e. the marker is in LD with a susceptibility locus, (3) Natural selection, i.e. there is selection in favor of a certain allele of the marker (4) Population stratification, i.e. the marker allele frequencies are different in the patient and control groups due to population stratification or (5) a False-positive result due to multiple statistical tests.

4.3.5 Linkage disequilibrium (LD) analysis

LD is defined as the non-random association of marker alleles (Slatkin 1994). LD can arise because of several causes, including (1) recent mutation, (2) population founder effects, (3) recent admixture of populations with different allele frequencies and (4) selection in favour of a specific allele (Slatkin 1994). LD mapping is typically used after linkage analysis in mapping monogenic disorders**,** but genome-wide scans using LD have also been regarded as the method of choice for mapping complex traits (Lander and Schork 1994; Kruglyak 1999; Reich et al. 2001).

4.3.6 Genome-wide association studies

Genome-wide association studies are suggested to be the most efficient way to investigate complex traits (Lander and Schork 1994; Kruglyak 1999; Reich et al. 2001). One reason for this is that association tests are likely to be more powerful than linkage analysis for detecting alleles of modest effect (Risch and Merikangas 1996). New technologies and experimental resources are making whole-genome association studies more feasible, and thus, future approaches to identify genes for complex diseases will include genome-wide association studies. Although whole-genome association studies have good prospects for dissecting the genetics of common diseases, they are currently facing a number of challenges, including problems of multiple testing and study design, definition of intermediate phenotypes and interaction between polymorphisms (Carlson et al. 2004).

4.4 Haplotype blocks and the HapMap project

After fine mapping the region using multiallelic markers and SNPs, haplotypes can be constructed and monitored. The density of markers required depends on the length of LD in the population investigated. Haplotype blocks may help LD mapping by allowing sparser marker maps (Gabriel et al. 2002). The haplotype blocks are sizable regions over which there is little evidence for historical recombination. These haplotype blocks show limited diversity and, in fact, only a few common haplotypes account for over 80% of all haplotypes in the block (Daly et al. 2001; Patil et al. 2001; Gabriel et al. 2002). Therefore, instead of genotyping all SNPs within the haplotype block, only a small fraction, defined as tagging SNPs, are needed to capture most of the haplotype diversity in the population. Such haplotype frameworks, within which only a few common haplotypes are observed, might provide substantial statistical power in association studies of common genetic variation across each region in the future (Gabriel et al. 2002). The constructed haplotype maps of the human genome (www.hapmap.org) may facilitate comprehensive genetic association studies of human disease. However, the optimal SNP density for genome-wide and regional association studies remains to be determined.

The HapMap project (www.hapmap.org) is currently building the haplotype blocks in the human genome. The project is expected to take three years and it involves 9 research groups in 5 countries. The aim is to analyze the block patterns in blood samples taken from people in Japan, Nigeria, and China and from people of northern and western European ancestry in the United States. The HapMap project aims to clarify how the human genome is organized into haplotype blocks. The main purpose is that mapping these haplotypes may form a powerful shortcut to identifying genes for complex traits in the future. The project is largely based on the theory of common variants underlying common disorders (CV/CD hypothesis) (Reich and Lander 2001).

The HapMap is a controversial project among human geneticists because of the questions regarding the block theory. The recent studies (reviewed in Wall and Pritchard 2003) indicate that the structure of LD is "blocklike" in some regions of the genome but not in others, thus some genomic regions fit better to the block theory than others. To summarize, usefulness of the haplotype blocks for future association studies will be uneven across the genome and will depend on the specific patterns of LD in the region of interest (Wall and Pritchard 2003).

With the availability of the human genome sequence and an increasing number of genomic sequences of other species, sequence-based gene discovery will eventually replace the traditional map-based gene discovery (positional cloning) (Peltonen and McKusick 2001). Two future approaches in discovering genetic variations underlying complex disease are a genomic-scale sequence-based approach and a genome-wide haplotype map (utilization of haplotype blocks) (Botstein and Risch 2003). In the sequence-based approach it is assumed that functional SNPs reside in coding regions, splice junctions or promoter regions. It is also expected that nonconservative changes in conserved amino acids are likely to be functional. Thus, this approach can miss functional noncoding SNPs, except when they are evolutionarily conserved. Fewer genotyped SNPs are required as compared to the genome-wide haplotype map approach, and sequence-based strategy also has the potential to detect disease variants with low allele frequencies. Although the sequence-based approach will at least initially focus only on gene regions, which constitute less than 5% of total genomic DNA, it might yield much of the power needed to find the genes underlying complex diseases (Botstein and Risch 2003).

4.5 DNA microarray

Recently, a new technology, called DNA microarray (reviewed in Chittur 2004), has made it possible to monitor the whole genome on a single chip. In a microarray, DNA molecules representing thousands of individual genes are spotted on a microscope slide and thus, researchers can have a picture of the interactions among a considerable number of genes simultaneously. RNA is purified from cells of a particular type and the array is exposed to a labeled sample RNA, hybridized, and the identity/abundance of complementary sequences are determined. Two major application forms for the DNA microarray technology exist: identification of sequence (gene/gene mutation); and determination of expression level (abundance) of genes. Consequently, it is possible to detect which genes are turned on or off in diseased versus healthy human tissues by using microarray technology. The genes that are expressed differently in the two tissues may be involved in causing the disease.

Over the past few years, microarrays have become more widely available to the researchers due to increased commercial availability of custom and generic arrays, the development of robotic equipment that has enabled array printing, and established analysis facilities in research institutions. Currently, microarray technology is a widely employed application for high-throughput comprehensive analysis of gene expression, as well as for genetic analysis and investigations of genomic changes associated with disease.

5. GENETICS OF LOW HDL-C AND FCHL DISORDERS

5.1 Candidate genes of low HDL-C

The inverse association between HDL-C and atherosclerotic cardiovascular disease has long been recognized. Decreased HDL-C, which is the most common dyslipidemia found among patients with premature CHD, is usually combined with other lipid disorders, especially hypertriglyceridemia. Pure isolated familial hypoalphalipoprotemia (HDL-C < 10^{th} percentile) have been found in 4% of patients with premature CHD as a sole lipid disorder (Genest et al. 1992).

The first observation about the correlation between genetic low HDL-C and precocious atherosclerosis was described by Norum et al (Norum et al. 1982). Paradoxally, monogenic disorders causing low HDL-C do not necessarily correlate with premature CHD. For example, in hypoalphalipoproteinemia caused by mutations in APOA1C3A4, APOA2, LCAT or CETP, no clear association of the genetic defect with CHD have been observed (Assmann et al. 1993; Rader et al. 1993a). Patients with very low HDL-C levels caused by the Apo A-I_{Milano} variant did not have atherosclerotic disease but rather a reduced CHD risk (Franceschini et al. 1980). Moreover, single gene defects are likely to create very low HDL-C levels (HDL-C < 10 mg/dL) without other metabolic problems, while the majority of low HDL-C syndromes, which are caused by multiple genes, exhibit higher HDL-C levels (20-40 mg/dL), and are typically identified in CHD patients (Miller et al. 2003). In addition to CHD, patients having the common form of low HDL-C often exhibit other typical features of the metabolic syndrome as well.

Many genes affecting plasma HDL-C levels have been described (Table 8). However, as in other complex disorders, replications of the identified polymorphisms in other study samples and in different populations have not always been very successful (see Discussion, second paragraph). Interestingly, a recent study suggests that rare alleles with major phenotypic effects significantly influence low HDL-C levels in the general population (Cohen et al. 2004). To determine wheteher sequence variants collectively contribute to variation in plasma levels of HDL-C, they sequenced three candidate genes causing Mendelian forms of low HDL-C, ABCA1, APOA1 and LCAT, in individuals from a population-based study. In that study, nonsynonymous sequence variants were significantly more common in individuals with low HDL-C than in those with high HDL-C, suggesting that rare DNA sequence variants collectively contribute to variation in HDL-C levels.

Table 8. Genetic defects influencing HDL-C levels.

GENE	LOCUS	SYNDROME	HDL	REPLICATED (+)	REFERENCES
ABCA1	9q31.1	Tangier's disease, Familial HDL deficiency	↓	+	Bodzioch et al. 1999; Brooks-Wilson et al. 1999; Rust et al. 1999
LCAT	16q22.1	Fish-eye disease, LCAT deficiency	↓	+	Funke et al. 1991; Kuivenhoven et al. 1997; Miettinen et al. 1998
APOA1	11q23-q24	HDL deficiency, Familial hypoalphalipoproteinemia, Familial hyperalphalipoproteinemia	↓↑	+	Ordovas et al. 1986; Jeenah et al. 1990; Rader et al. 1993b; Miettinen et al. 1997; Yamakawa-Kobayashi et al. 1999 etc.
APOA2	1q21-q23	Familial apo-A-II deficiency	↔		Deeb et al. 1990
APOC3	11q23.1-q23.2	hypertriglyceridemia	variation		Gagnon et al. 2003
APOA1C3A4 gene cluster	11q23	ApoA-I/C-III deficiency, ApoA-I/C-III/A-IV deficiency	↓		Schaefer et al. 1985; Ordovas et al. 1989
APOA5	11q23	hypertriglyceridemia	↓		Li et al. 2004
LIPC (HL)	15q21-q23	Hepatic lipase deficiency	↑	+	Guerra et al. 1997; Jansen et al. 2002; Su et al. 2002
LPL	8p22	Familial Chylomicronemia syndrome, FCHL, Hyperlipoproteinemia I, Familial LPL deficiency	↓	+	Pimstone et al. 1995; Reymer et al. 1995a
PLTP	20q12-q13.1	No described syndromes in human	↓*		Jiang et al. 2001
CETP	16q21	CETP deficiency	↑		Brown et al. 1989; Zhong et al. 1996; Barzilai et al. 2003
SCARB1 (SR-B1)	12q24.31	No described syndromes in human	↑		Rigotti et al. 1997; Acton et al. 1999

*In mice

5.2 Candidate genes of FCHL

Although FCHL was originally suggested to be a monogenic trait (Goldstein et al. 1973), the inheritance of the FCHL-associated lipid phenotype has later been shown to be complex (Kwiterovich 1993; Aouizerat et al. 1999a). Multiple genes have been shown to play an important role in FCHL and to affect the component lipid traits characteristic of this disorder. Candidate genes of FCHL are shown in Table 9. Juo and colleagues suggested that a common genetic mechanism determines plasma apoB levels and small dense LDL subfraction distribution in FCHL, and a major gene that has pleiotropic effects on these lipid parameters could be the gene underlying FCHL (Juo et al. 1998).

Table 9. Candidate genes of FCHL.

GENE	LOCUS	REPLICATED (+)	REFERENCES
APOA1C3A4-A5 gene cluster	11q13-qter	+	Wojciechowski et al. 1991; Xu et al. 1994; Dallinga-Thie et al. 1996; Deeb et al. 1996; Groenendijk et al. 1999; Groenendijk et al. 2001b; Groenendijk et al. 2001c; Groenendijk et al. 2001d; Eichenbaum-Voline et al. 2004; Mar et al. 2004 etc.
APOA2	1q21-q23		Allayee et al. 2003
APOE	19q13.2		Bredie et al. 1996
LPL	8p22	+	Gagne et al. 1994; Reymer et al. 1995b; de Bruin et al. 1996; Hoffer et al. 1996; Hoffer et al. 1998; Campagna et al. 2002
LIPC (HL)	15q21-q23	+	Gehrisch et al. 1999; Allayee et al. 2000
FABP2	4q28-q31		Pihlajamaki et al. 1997
MNSOD	6q25.3		Allayee et al. 1998; Aouizerat et al. 1999b
LDL-R	19p13.3		Allayee et al. 1998
CETP	16q21		Allayee et al. 1998
LCAT	16q22.1		Allayee et al. 1998; Aouizerat et al. 1999b
USF1	1q22-q23		Pajukanta et al. 2004
TNFRSF1B	1p36.3-p36.2		Geurts et al. 2000

FABP2 indicates the gene encoding Intestinal fatty acid binding protein 2-gene, MNSOD the gene encoding Manganese superoxide dismutase, TNFRSF1B the gene encoding the tumor necrosis factor receptor superfamily, member 1B. APOA1C3A4A5 indicates genes encoding apolipoproteins A-I C-III A-IV and A-V.

Several segregation analysis studies have provided evidence of a gene for elevation of apoB levels (Bredie et al. 1997b). In Dutch FCHL families, evidence for linkage with elevated apoB levels and a locus on chromosome 1p31 was reported (Allayee et al. 2002). A number of variants of the APOA1C3A4 gene cluster (Hayden et al. 1987; Wojciechowski et al. 1991; Groenendijk et al. 2001b; Groenendijk et al. 2001c; Groenendijk et al. 2001d) have also been implicated in the development of FCHL.

Variations in genes affecting the removal rate of TGs from plasma, such as the LPL gene, have been shown to influence the lipid phenotypic expression of FCHL: FCHL individuals with the N291S variant in the LPL gene showed higher plasma lipids and apoB levels compared with affected non-carriers (Campagna et al. 2002). The same variant was associated with decreased HDL-C and increased TG levels in males with FCHL in a study of Reymer et al. (Reymer et al. 1995b), indicating that defective LPL may be one of the factors contributing to the FCHL phenotype. Similarly, two mutations in the LPL gene have been associated with higher plasma TG in FCHL patients in an other study (de Bruin et al. 1996). However, in French Canadian and Finnish subjects with FCHL, the LPL gene mutations showed no association (Marcil et al. 1996; Pajukanta et al. 1997).

5.3 Some important candidate genes of low HDL-C and FCHL

ABCA1

ATP-binding cassette transporter A 1 (ABCA1) is a membrane protein that mediates cholesterol efflux from e.g. macrophages to HDL particles, and thus, takes part in reverse cholesterol transport. The ABCA1 gene resides on chromosome 9q31.1 and is expressed in the liver, the macrophages, and the steroidogenic tissues.

Mutations in the ABCA1 gene cause Tangier disease, a rare autosomal recessive disorder with severe HDL deficiency (Bodzioch et al. 1999; Brooks-Wilson et al. 1999; Rust et al. 1999). Tangier disease has been diagnosed in approximately 40 patients worldwide. In addition to almost complete absence of HDL-C, typical features of Tangier disease include accumulation of cholesterol esters, which leads to enlarged yellow tonsils, hepatosplenomegaly, peripheral neuropathy, and frequently premature CHD (Assmann et al. 1995).

Interestingly, mutations in the ABCA1 gene were also found in French-Canadian patients with familial HDL-C deficiency (Brooks-Wilson et al. 1999). However, a population study of familial HDL deficiency in Finns found no significant association between ABCA1 polymorphism and low HDL-C (Kakko et al. 2003). It is worth noting that familial hypoalphalipoproteinemia patients with ABCA1 mutations have considerably lower HDL-C levels than average CHD patients (Miller et al. 2003). This suggests that HDL-C deficiency caused by ABCA1 variants accounts for only a minority of such cases, and thus, the ABCA1 gene is unlikely to be a major regulator of HDL-C in the general population.

APOAIC3A4 and APOA5

The relationship between variations in the APOAIC3A4 gene cluster and several plasma lipid abnormalities has been well recognized for a long time (reviewed in Groenendijk et al. 2001a). Thus, APOAIC3A4 has been an attractive candidate gene for both FCHL and low HDL-C. Multiple studies predict the importance of the apoAI-CIII-AIV gene cluster as a modifier gene complex in the development of FCHL (Hayden et al. 1987; Wojciechowski et al. 1991; Xu et al. 1994; Dallinga-Thie et al. 1996; Dallinga-Thie et al. 1997; Groenendijk et al. 1999). For example, APOAIC3A4 has been shown to affect LDL-C and apolipoproteins B and C-III (Dallinga-Thie et al. 1996).

The most studied polymorphisms of the APOAIC3A4 gene cluster include three restriction enzyme polymorphisms, XmnI and MspI residing on sites 5' of the APOA1 gene, and the SstI site in the 3' untranslated region of exon 4 of the APOC3 gene (for review see Groenendijk et al. 2001a). The minor alleles of these polymorphisms were associated with elevated plasma cholesterol, TGs, LDL-C, apoB, and apoC-III levels in Dutch FCHL families (Dallinga-Thie et al. 1996). Furthermore, suggestive evidence for linkage between the MspI minor allele and plasma LDL cholesterol levels was detected (Dallinga-Thie et al. 1996). Based on the results obtained in this study, Dallinga-Thie et al. suggested, that the APOAIC3A4 gene cluster is not the primary cause of FCHL, but rather it has a specific modifying effect on plasma triglyceride and LDL cholesterol levels in this lipid disorder (Dallinga-Thie et al. 1996).

In Finnish FCHL families, the MspI polymorphism of the APOA1 gene was associated with total serum cholesterol and apoB levels in spouses, but no evidence of direct involvement of the APOAIC3A4 loci or haplotypes in the expression of FCHL was found (Tahvanainen et al. 1998). Likewise, in French-Canadian subjects with FCHL, no associations were detected between the APOAIC3A4 gene XmnI and SstI polymorphisms (Marcil et al. 1996).

The APOA1C3A4 gene complex has been associated with HDL-C levels in multiple studies (Table 8). Numerous mutations in APOA1 have been described, usually causing decreased HDL-C levels (Ordovas et al. 1986; Yamakawa-Kobayashi et al. 1999), but mutations causing familial hyperalphalipoproteinemia have also been reported (Jeenah et al. 1990; Rader et al. 1993b).

Quite recently, a new member of the APOAIC3A4 gene cluster was proposed: APOA5 (Pennacchio et al. 2001). Since then, growing evidence of the role of APOA5 as an important determinant of plasma TG and cholesterol has been demonstrated, and it seems that APOA5 is also a potent risk factor for cardiovascular disease (Aouizerat et al. 2003; Pennacchio and Rubin 2003a). ApoAV is suggested to reduce plasma triglycerides by inhibiting lipidation of apoB and thus reducing the hepatic VLDL production rate as well as by stimulating LPL-mediated clearance of TG-rich lipoproteins (Schaap et al. 2004).

A strong genetic association has been described between APOA5 polymorphisms and TG levels (Endo et al. 2002; Ribalta et al. 2002; Talmud et al. 2002; Aouizerat et al. 2003; Baum et al. 2003; Martin et al. 2003b; Masana et al. 2003; Seda and Sedova 2003). Over-expression of APOA5 has been shown to lower TGs in mice and APOA5 knockout mice have severe hypertriglyceridemia (van der Vliet et al. 2002). APOA5 is a highly responsive peroxisome proliferator-activated receptor alpha target gene and may act as a major mediator for fibrates in reduction of plasma triglycerides (Vu-Dac et al. 2003).

Linkage and association between variants of the APOA1C3A4A5 gene cluster and FCHL have been demonstrated recently (Eichenbaum-Voline et al. 2004). In more detail, two independent alleles, APOA5c.56G (i.e. S19W) and APOC3c.386G, of the APOA1C3A4A5 gene cluster were overtransmitted in FCHL. In a study of Mar et al., APOA1C3A4A5 showed association with TG levels and LDL particle size in Dutch FCHL families (Mar et al. 2004). The strongest evidence of association in that study was obtained with SNPs in APOA1 (XmnI, MspI) and APOA5 (S19W).

APOA2

The exact function of the second most abundant protein component of HDL-C, ApoA-II, is still unknown. However, polymorphisms in APOA2 have been associated with moderate effects on HDL-C level and/or composition in humans (Scott et al. 1985; Bu et al. 1994; Brousseau et al. 2002; Han et al. 2002). In mice, APOA2 has been associated with HDL-C levels (Hedrick et al. 1993; Mehrabian et al. 1993).

In FCHL, both biochemical and genetic association of plasma apoA-II levels have been suggested: FCHL patients had higher apoA-II levels compared with unaffected relatives, and apoA-II levels showed significant evidence for linkage (LOD=3.1) to a locus on chromosome 1q41 in Dutch FCHL families (Allayee et al. 2003). However, APOA2 is not likely to be a primary genetic determinant of FCHL, as discussed recently by Aouizerat and Kane (Aouizerat and Kane 2003), because the lack of major linkage evidence to the APOA2 region in previous studies (Aouizerat et al. 1999c; van der Kallen et al. 2000). Also in Finnish FCHL families linked to 1q21-23, the peak linkage signal was produced by a marker located about 5 cM from APOA2 (Pajukanta et al. 1998), and association was later detected with variations of the USF1 gene (IV).

TXNIP

A gene for combined hyperlipidemia (Hyplip1) in the mouse has been mapped to a region on mouse chromosome 3 that is potentially orthologous to human chromosome 1q21 (Castellani et al. 1998). The chromosome 1q21 has been identified as an FCHL locus in Finnish, Dutch, German, Chinese and US families (Pajukanta et al. 1998; Coon et al. 2000; Pei et al. 2000; Allayee et al. 2002). The underlying gene, thioredoxin interacting protein gene (TXNIP), which encodes a cytoplasmic protein that inhibits thioredoxin, a regulator of the cellular redox state (Bodnar et al. 2002), thus provided a strong positional candidate for human FCHL (Pajukanta et al. 2001). However, no association between the TXNIP gene and FCHL was found in Finnish FCHL families (IV). Just recently, this negative result has been replicated in Dutch FCHL families (van der Vleuten et al. 2004) as well as in FCHL families participating in the NHLBI Family Heart Study (Coon et al. 2004). Thus, it seems that TXNIP does not play a major role in human FCHL or related traits, and it is unlikely to account for the positive evidence of linkage between FCHL and the 1q21 region.

5.4 Chromosomal loci identified for low HDL-C

Recent family studies have identified six novel loci for this complex disorder (Table 10): Kort et al. found evidence for linkage of the hypoalphalipoproteinemia trait to chromosome 11q23.3 in 105 large Utah pedigrees, approximately 10 cM distal to the apolipoprotein A-I/C-III/A-IV gene cluster (APOA1C3A4) (Kort et al. 2000). In 10 large, randomly ascertained Mexican American families, two novel loci influencing HDL-C levels were localized on chromosomes 8 and 15, using QTL mapping strategy (Almasy et al. 1999). The underlying genes have not yet been identified. A linkage of HDL-cholesterol concentrations to a locus on chromosome 9p has also been demonstrated in Mexican Americans (Arya et al. 2002). The fifth potential locus is the ABCA1 gene on chromosome 9, mutated in the monogenic Tangier disease. ABCA1 can also have allelic variants predisposing to common forms of familial HDL deficiency, as exemplified in

four French-Canadian families with hypoalphalipoproteinemia (Brooks-Wilson et al. 1999). Most recently, a quantitative trait locus influencing plasma HDL-C levels was detected on chromosome 16q in Mexican Americans (Mahaney et al. 2003). This novel QTL locus for HDL-C levels on 16q resides near the loci for LCAT and cholesterol ester transfer protein (CETP). Further evidence of involvement of the 16q locus with a low HDL-C trait has been obtained in a combined data analysis in Finnish and Dutch FCHL families (Pajukanta et al. 2003). In that study, low HDL-C, a component trait of FCHL, was also found to be linked to 9p23.

Table 10. Six potential loci identified for common forms of low HDL-C. Loci marked in bold have been replicated in other study samples.

LOCUS	STUDY SAMPLE	REFERENCES
8q24	Mexican Americans	Almasy et al: 1999
9q31 (ABCA1 gene)	French-Canadian	Brooks-Wilson et al: 1999
11q23	Utah families	Kort et al: 2000
15q21	Mexican Americans	Almasy et al: 1999
16q	Mexican Americans	Mahaney et al. 2003
16q24.1	Finnish and Dutch families	Pajukanta et al. 2003

5.5 Chromosomal loci identified for FCHL

So far, three genome-wide scans have been performed for FCHL in the Finnish (Pajukanta et al. 1999), Dutch (Aouizerat et al. 1999c) and British FCHL families (Naoumova et al. 2003). These scans have identified several chromosomal regions for the FCHL trait or its component traits (Table 11).

Table 11. Loci identified for FCHL in genome-wide scans of FCHL families originating from different populations. Loci that have been detected in several FCHL study samples are indicated in bold.

STUDY SAMPLE	LOCI	REFERENCES
Finnish	**1q21, 2p**, 2q, **9p**, **10p**, 10q, **16q**, 20q, 21q	Pajukanta et al. 1998 & 2003, Soro et al. 2002
Dutch	1p, **1q21, 2p, 9p, 11p, 16q**, 19q	Aouizerat et al. 1999, Allayee et al. 2002, Pajukanta et al. 2003
British	6q, 8p, **11p**	Naoumova et al. 2003

Some FCHL loci have been replicated in several study samples (loci marked in bold in Table 11). For example, the 1q21 locus has been detected in both Finnish and Dutch FCHL families, as well as in US Caucasians, Chinese and German study samples (Pajukanta et al. 1998; Coon et al. 2000; Pei et al. 2000; Allayee et al. 2002). Another replicated example is the 11p region, which has been detected separately in Dutch and British FCHL families (Aouizerat et al. 1999c; Naoumova et al. 2003). Still, given the expected difficulties in replicating and verifying the results of a genome-wide scan, international collaboration to combine study samples is the utmost importance to accelerate the identification of genes for common cardiovascular traits such as FCHL. This strategy is based on increased statistical power and has been used in previous studies

with other complex traits and it has even led to gene identification in recent studies of asthma (Van Eerdewegh et al. 2002) and inflammatory bowel disease (Hugot et al. 2001; Ogura et al. 2001). This approach was also successfully utilized in a previous pooled data analysis of the Dutch and Finnish FCHL genome scans to identify shared chromosomal regions for FCHL and its component traits (Pajukanta et al. 2003). After unifying the diagnostic criteria and pooling the genotype data, three regions were identified on chromosomes 16q, 2p, and 9p. The 16q24.1 region produced a parametric multipoint LOD score of 3.4 using a parametric location score analysis for the low HDL-C trait. There was also evidence for locus heterogeneity among the families, with about half of them exhibiting linkage to this chromosomal region. Since then, this very same region on 16q has also been linked to HDL-C in an independent study of Mexican Americans (Mahaney et al. 2003).

5.6 Chromosome 1q21 region

The first major locus for FCHL was identified in Finnish FCHL families on chromosome 1q21-23 in 1998 (Pajukanta et al. 1998). This was the first novel locus for FCHL detected since it was described in 1973 (Goldstein et al. 1973). To date, FCHL linkage to 1q21-23 has been replicated in FCHL families originating from many other, more heterogeneous populations (Coon et al. 2000; Pei et al. 2000; Allayee et al. 2002) (Table 12).

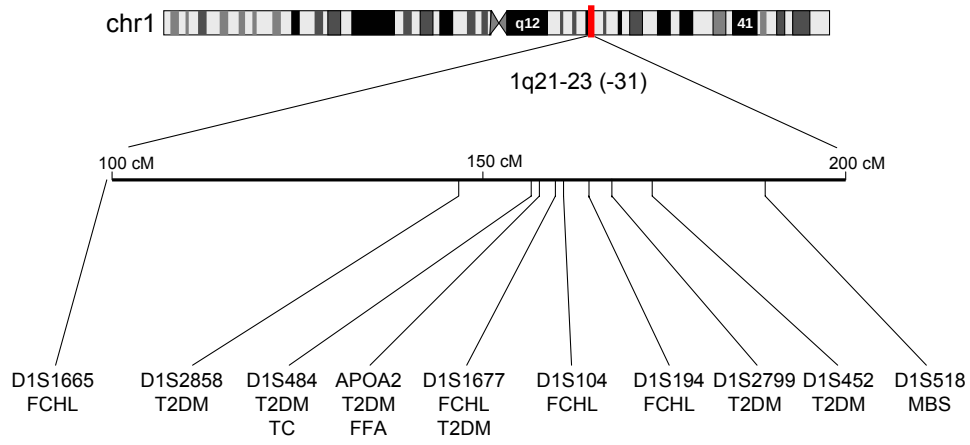
Table 12. Traits linked to 1q21-23 (-31)

TRAIT	STUDY SAMPLE	PEAK MARKER/ LOCATION	REFERENCES
FCHL	Finns	D1S104 161,9 cM*	Pajukanta et al. 1998
	U.S. Caucasians	D1S104-D1S1677 161,9-161,5 cM*	Coon et al. 2000
	Germans	D1S194 165.9 cM*	Pei et al. 2000
	Chinese	D1S194 165,9 cM*	Pei et al. 2000
	Dutch	D1S1665 99,6 cM*	Allayee et al. 2002
Type 2 DM	Pimas	D1S1677 161,5 cM*	Hanson et al. 1998
	Utah Caucasians	CRP-APOA2 (165.6–170.8 cM)	Elbein et al. 1999
	French	APOA2-D1S484 158,0-157,5 cM*	Vionnet et al. 2000
	British	D1S2799-D1S452 169,7- 173,2 cM*	Wiltshire et al. 2001
	Amish	near D1S2858 147,2 cM*	Hsueh et al. 2003
	Finns	165 cM	Watanabe et al. 2000
	Chinese	D1S1589 176,25 cM*	Xiang et al. 2004
Metabolic syndrome	Hispanic	D1S518 188,0 cM*	Langefeld et al. 2004
Cholesterol	Caucasians	D1S484 157,5 cM*	Reed et al. 2001
Lipoprotein(a)	Western Europeans	150-200 cM	Broeckel et al. 2002
Plasma FFA	Caucasians	APOA2 158,0 cM	Warden et al. 1993

*Estimated sex-averaged genetic location from the p-telomere in centiMorgans according to the deCODE map (http://www.decode.com/)

Interestingly, the same markers in the 1q21 region have also been linked to type 2 DM in numerous studies (Hanson et al. 1998; Elbein et al. 1999; Watanabe et al. 2000; Vionnet et al. 2000; Wiltshire et al. 2001; Hsueh et al. 2003), including a Finnish study (Watanabe et al. 2000) (Table 12, Figure 7). Most recently, the very same chromosomal region has been linked to the metabolic syndrome (Langefeld et al. 2004). Therefore, different allelic variants or haplotypes of the same gene may underlie the FCHL, type 2 DM, and the metabolic syndrome linkage results. In addition to overlapping linkage results, these traits share many of their critical metabolic features, further supporting a hypothesis of the partially shared genetic background between these complex disorders.

Figure 7. The critical FCHL area on 1q21-23 and the markers of which have been linked to FCHL, type 2 DM, free fatty acids (FFA), total cholesterol (TC), and the metabolic syndrome (MBS).



AIMS OF THE PRESENT STUDY

The following specific aims were addressed in this study:

(I) To study the contribution of 21 known candidate genes of HDL-C metabolism for the trait in Finnish low HDL-C families.

(II) To perform a genome-wide scan to identify novel susceptibility loci for low HDL-C.

(III) To further investigate the chromosomal loci identified previously in Finnish cardiovascular study samples and to determine the involvement of quantitative lipid traits in Finnish low HDL-C and FCHL families.

(IV) To fine map and identify the FCHL gene on chromosome 1q21-23.

MATERIALS AND METHODS

1. TABLE OF MATERIALS AND METHODS

The details of the methods used in this study are described in the original publications (I-IV) according to Table 13 below.

Table 13. Materials and methods used in this study.

Material or method	Original publication
Study samples	
Low HDL-C families	I-III
FCHL families	II-IV
Cases and controls	I
Coding as affected subjects	I-IV
DNA extraction	I
PCR and sequencing	I, IV
Statistical analyses	I-IV
Bioinformatics	I-IV
Biochemical analyses	I-III
Genome-wide scan	II
Candidate gene approach	I
Genotyping	I-III
Marker and SNP selection	I-IV
Marker maps	I-IV
Fat biopsies and RNA extraction	IV
Expression array analysis of adipose tissue	IV
Bioinformatics	I-IV
Quantitative real-time PCR analysis	IV
Reporter gene analysis for the transcription efficiency	IV

2. SUBJECTS

The Finnish low HDL-C families were recruited in the Helsinki and Turku University Central Hospitals. The Finnish FCHL families were recruited in the Helsinki, Turku and Kuopio University Central Hospitals. The family collection consisted of three phases. In the first phase, probands were selected from patients undergoing elective coronary angiography or from a registry of patients with confirmed myocardial infarction. In the second phase, probands and all the first-degree relatives were studied to identify low HDL and FCHL families. Finally, in the third phase, all accessible relatives and spouses were contacted and examined, and extensive metabolic and genetic studies were performed. Each subject provided a written informed consent prior to participating in the study. All samples were collected in accordance with the Helsinki declaration, and the ethics committees of the participating centers approved the study designs presented in this thesis.

2.1 Study sample 1: Low HDL-C families (I-III)

Table 14. Diagnostic criteria for low HDL-C probands

INCLUSION CRITERIA
Age 30-60 years
HDL-C < 10th age-sex specific Finnish population percentiles
Premature CHD (angiographically verified >50% stenosis at least in one coronary artery or survived myocardial infarction)
At least three accessible first-degree relatives
At least one first degree relative affected by low HDL-C
EXCLUSION CRITERIA
Diabetes mellitus, type 1 and type 2
BMI >30 kg/m^2
Severe renal or hepatic disease
Hypertriglyceridemia (TG > 2.3 mmol/l for both genders)
Hypercholesterolemia (TC > 6.3 mmol/l ♂, > 6.0 mmol/l ♀)

The following three criteria had to be fulfilled for the myocardial infarction diagnosis: (1) typical clinical symptoms, (2) definite electrocardiographic findings, according to the Minnesota coding (WHO criteria) (Rose et al. 1982), and (3) elevated levels of the creatine-kinase enzyme (CK) and its cardiac isoenzyme, CK-MB.

If the proband fulfilled the criteria mentioned above, families with at least two affected members were included in the study and all the accessible family members were examined. The affected family members were ascertained for low HDL-C, using the Finnish age-sex specific 10th population percentiles (Table 15) derived from the population-based surveys FINRISK and LASERI (Porkka et al. 1994; Vartiainen et al. 1994; Vartiainen et al. 2000).

Table 15. The Finnish age-sex specific 10th population percentiles for HDL-C (mmol/l).

Age (year)	Males	Females
5-11	1.0	1.1
12-14	1.0	1.1
15-17	0.9	1.0
18-20	0.9	1.1
21-24	0.8	1.1
25-29	0.9	1.1
30-34	0.9	1.1
35-39	0.9	1.1
40-44	0.9	1.1
45-49	0.9	1.1
50-54	0.9	1.1
55-59	0.9	1.1
60-	0.9	1.1

2.2 Study sample 2: Finnish FCHL families (II-IV)

Table 16. Diagnostic criteria for FCHL probands

INCLUSION CRITERIA
Age 30-55 years ♂, <60 years ♀
TC and/or TGs >age-sex specific Finnish 90th percentiles
Premature CHD (angiographically verified >50% stenosis in at least one coronary artery or survived myocardial infarction)
At least one first degree relative affected by FCHL (high TC and/or high TGs)[1]
EXCLUSION CRITERIA
Diabetes mellitus, type 1[2]
Severe renal or hepatic disease
Hypothyroidism
Familial hypercholesterolemia[3]

[1]If the proband had only one elevated lipid trait, a first-degree relative had to have the combined phenotype.
[2]Type 2 DM and obesity were not exclusion criteria in the Finnish FCHL families.
[3]Familial hypercholesterolemia was excluded by determining the LDL-receptor status of the proband by the lymphocyte culture method (Cuthbert et al. 1986).

If the criteria mentioned above were fulfilled, families with at least two affected members were included in the study, and all the accessible family members were examined. FCHL and TGs traits were analyzed. For the FCHL trait, family members were scored as affected if they had the combined hyperlipidemia phenotype or if they had high TC or high TGs using the Finnish age-sex specific 90th percentiles (Table 17). These ascertainment criteria are fully comparable with the original FCHL criteria (Goldstein et al. 1973). For dichotomized analysis of TGs, family members with TG levels $\geq$ 90th Finnish age-sex specific population percentile were coded as affected, and family members with < 90th percentile as unknown.

Table 17.
The 90th age-sex specific population percentiles for total cholesterol (TC), triglycerides (TGs) and apolipoprotein B (apoB) in the Finnish population.

	TC (mmol/l)	TC (mmol/l)	TGs (mmol/l)	TGs (mmol/l)	ApoB (mg/dl)	ApoB (mg/dl)
Age (y)	Men	Women	Men	Women	Men	Women
5-11	5.9	6.2	1.6	1.4	100	108
12-14	6.0	5.8	1.7	1.6	104	103
15-17	5.3	5.6	1.9	1.5	92	101
18-20	5.9	6.0	2.0	1.7	111	111
21-24	5.7	5.9	2.3	1.6	109	115
25-29	6.2	6.1	2.4	1.7	107	102
30-34	6.6	6.2	2.7	1.7	114	106
35-39	7.0	6.4	2.9	1.8	120	109
40-44	7.2	6.6	3.2	1.8	124	112
45-49	7.4	6.8	3.4	1.9	127	115
50-54	7.5	7.1	3.5	2.1	129	119
55-59	7.5	7.3	3.5	2.3	128	122
60->	7.4	7.6	3.5	2.5	127	125

Worth noting is the fact that type 2 DM and obesity were not exclusion criteria for the probands of the families with FCHL. Because of this, some patients fulfilling the criteria of the metabolic syndrome also exist in the study sample.

3. LIPID MEASUREMENTS

All blood samples for the measurement of serum lipids and for DNA isolation were obtained after an overnight fast. Any lipid lowering medication was interrupted for four weeks before the blood samples were taken. All lipid measurements for low HDL and FCHL study subjects were performed according to the same protocols, and moreover, in the same research laboratory of the Division of Cardiology, Department of Medicine, Helsinki University Central Hospital. However, lipid measurements of 6 FCHL families recruited in Kuopio Central Hospital were performed in Kuopio. Serum TC and TGs were measured with an automated Cobas Mira analyser (Hoffman-La Roche, Basle, Switzerland) by enzymatic methods (Hoffman-La Roche kits 0722138 and 0715166, respectively). LDL was calculated using the Friedewald formula (Friedewald et al. 1972):

LDL-C = TC - HDL-C – (TG/2.2)

(Valid if TG < 4.5 mmol/L, no apo E2/2 genotype or phenotype, no familial dysbetalipoproteinemia)

Serum HDL-C was quantified by phosphotungstic acid/magnesium chloride precipitation procedures (Hoffman-La Roche kit 0720674). HDL2 and HDL3 were separated by sequential flotation in an ultracentrifuge (Taskinen et al. 1988). Concentrations of Apo-AI, Apo-AII and apoB were measured by immunoturbidimetric methods with commercial kits (Boehringer-Mannheim, Mannheim, Germany). Plasma glucose concentrations were analyzed by the glucose dehydrogenase method (Precision-G Blood Glucose Testing

System, Medisense, Abbott, Illinois, USA). Serum-free insulin concentrations were measured by radioimmunoassay (Phadeseph INSULIN RIA, Pharmacia & Upjohn, Uppsala, Sweden). The homeostasis model assessment for insulin resistance (HOMA IR) was calculated from the fasting plasma glucose and serum insulin concentrations as follows: fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5 (Matthews et al. 1985; Haffner et al. 1997).

RESULTS

1. CANDIDATE GENE STUDY OF FINNISH LOW HDL-C FAMILIES (I)

The specific aim was to identify and characterize candidate genes affecting the low HDL-C trait in the Finnish low HDL-C families. We analyzed 21 potential candidate genes (Table 18) with 48 flanking markers in 21 low HDL-C families. An additional 4 families were genotyped with the markers for the APOA2 and APOA1C3A4 gene cluster, which showed some evidence of linkage.

Table 18. Genes studied in the candidate gene project of low HDL-C.

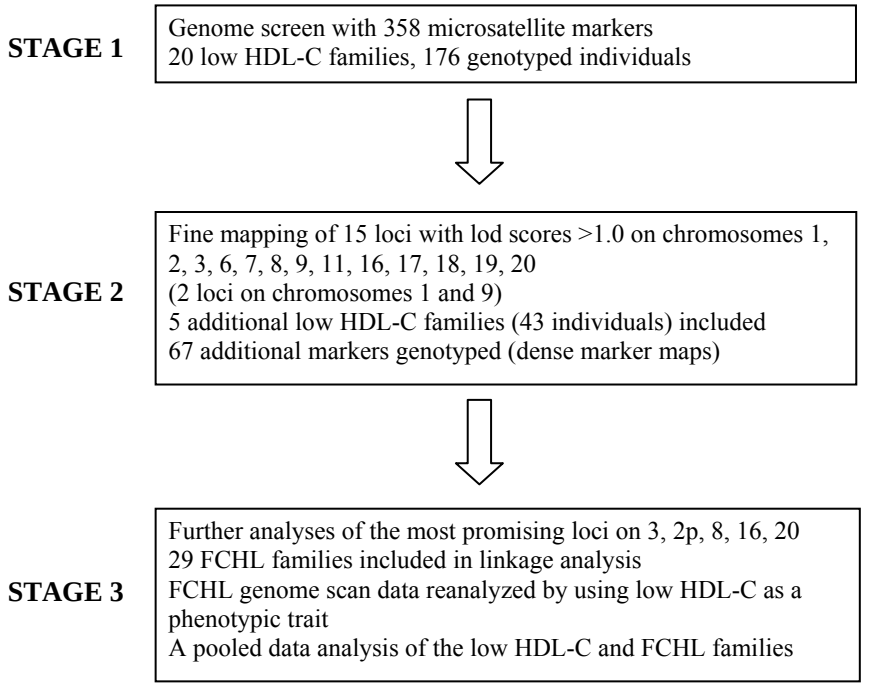
CANDIDATE GENE	ABBREVIATION	LOCATION	MARKERS
Apolipoprotein A-I/ C-III/ A-IV gene cluster	APOA1C3A4	11q23	D11S1347, Xmn1, Msp1, Sst1, D11S939
Apolipoprotein A-II	APOA2	1q21-q23	D1S2635, APOA2, D1S2844, D1S104
Apolipoprotein C-II	APOC2	19q13.2	D19S178, APOC2, D19S219
Cholesterol ester transfer protein	CETP	16q13	D16S3057, D16S3143, D16S514
Hepatic lipase	HL, LIPC	15q21-q22	D15S117, LIPC, D15S98
Lecithin:cholesterol acyltransferase	LCAT	16q22.1	D16S3031, D16S496, D16S3139
Phospholipid transfer protein	PLTP	20q12-q13.1	PPGPR, PPGB.PCR
Peroxisome proliferator activated receptor α	PPARα	22q12-q13.1	D22S928, D22S1160
Peroxisome proliferator activated receptor γ	PPARγ	3p25	D3S1259, D3S1585, D3S1286
Macrophage scavenger receptor	MSR	8p22	D8S1731, D8S261
Lipoprotein lipase	LPL	8p22	D8S282
HDL-binding protein	HDLBP	2q37	D2S125, D2S395, D2S338
Alpha-1-antitrypsin	A1AT	14q32.1	D14S265, D14S1142
Selectins P, L and E	SELP, SELL, SELE	1q23-25	D1S196, SELP, D1S210
ATP-binding cassette transporter A 1	ABCA1	9q31	D9S277, D9S306
Endothelial lipase	LIPG	18q21.31-q21.33	D18S460, D18S118, D18S1145
Scavenger receptor B 1	SR-B1	12q24.31-q24.32	D12S340, D12S1714, D12S97

In this candidate gene project**,** we found suggestive evidence for linkage with two loci: Marker D1S2844, located in the APOA2 region, yielded a LOD score of 2.14 and marker D11S939 flanking the APOA1C3A4 gene cluster produced a LOD score of 1.69. We also identified potential shared haplotypes in these two regions in a subset of low HDL families. These results suggest that APOA2 and APOA1C3A4 genes or genes in their immediate vicinity are likely to have an influence on HDL levels. It is conceivable that the obtained linkage result to APOA2 locus on chromosome 1 may as well reflect the presence of the near-by located USF1 gene linked to FCHL in Finnish FCHL families (IV).

2. GENOME-WIDE SCAN IN FINNISH LOW HDL-C FAMILIES (II)

The specific aim of this study was to identify new loci and finally novel genes regulating the metabolic pathways determining serum HDL levels in the Finnish population. We performed a genome-wide scan in three stages (Figure 8). At stage 1, 358 microsatellite markers were genotyped in 20 low HDL-C families. At stage 2, 67 additional markers were genotyped for the regions showing lod scores > 1.0 in stage 1 (15 loci) and 5 low HDL-C families were added to the study sample. The fine mapping provided further support for linkage to the low HDL trait on chromosomes 2p ($Z=2.1$, $\theta=0.10$), 3p ($Z=2.1$, $\theta=0.\ 06$), 8q23 ($Z=2.3$, $\theta=0.10$), 16q24.1-24.2 ($Z=2.2$, $\theta=0.08$), and 20q13.11 ($Z=1.4$, $\theta=0.16$). The significance of these initial findings for low HDL-C was further addressed by analyzing markers in these regions in an independent study sample of 29 Finnish families with FCHL (stage 3), because low HDL-C forms one of the component traits of FCHL. This is also reflected by the fact that there were 64 individuals affected with low HDL-C in these FCHL families.

Figure 8. Overview of the strategy used in the genome-wide scan (study II).



The pooled data analysis of low HDL and FCHL study samples, using low HDL as a tested trait, provided further support for linkage in three of these regions on 8q23, 16q24.1-24.2 and 20q13.11 (Table 19). The highest statistical evidence for linkage was observed on chromosome 8q23 (a lod score of 3.9). Importantly, this locus has earlier been linked to HDL-C in Mexican Americans (Almasy et al. 1999). Further, the region on chromosome 20q has previously been linked to body adiposity, hyperinsulinemia and type 2 DM (Bowden et al. 1997; Ji et al. 1997; Zouali et al. 1997; Ghosh et al. 1999; Lee et al.

1999; Mohlke et al. 2001), suggesting a partially shared genetic background of these traits and HDL-C.

Table 19. The most significant results of the low HDL-C genome-wide search for low HDL-C trait obtained by analyzing the combined study sample of low HDL-C and FCHL families or by analyzing both family sets alone.

LOCUS*	MARKER	STUDY SAMPLE	LOD SCORE
2p25.1	D2S423	FCHL	3.4
2p13.2	D2S1394	low HDL-C	2.1
3p26.1	D3S1304	low HDL-C	2.1
8q23.1	D8S1132	combined	4.7
16q23.3	D16S3091	combined	2.2
20q13.32	D20S171	combined	1.9

* According to the July 2003 human reference sequence of UCSC Human Genome Browser Gateway (http://www.genome.ucsc.edu/)

3. FINE MAPPING OF SIX CARDIOVASCULAR LOCI IN FINNISH LOW HDL-C AND FCHL FAMILIES (III)

The aim of this study was to further investigate the significance of six cardiovascular loci identified previously in the genome wide scans of the Finnish study samples in an extended study material consisting of 92 low HDL and FCHL families. The analyzed loci were as follows: low HDL-C loci on 8q23, 16q24.1-24.2, and 20q13.11 (Soro et al. 2002), loci for premature CHD on 2q31 and Xq24 (Pajukanta et al. 2000) and a locus for serum TGs on 10q11 (Pajukanta et al. 1999) (Table 20). We fine mapped these loci and tested their involvement, especially in quantitative lipid traits in a combined study sample of 92 low HDL-C and FCHL families. A total of 67 microsatellite markers were genotyped for these six regions in 1109 individuals (Figure 9).

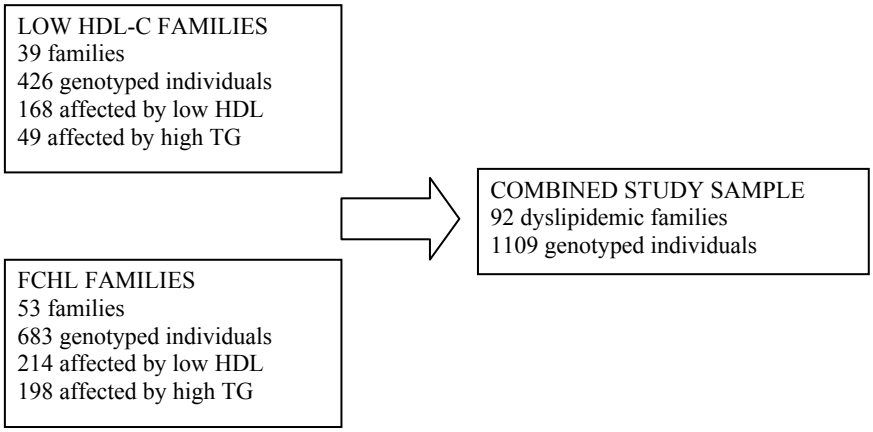
Table 20. Previous findings from three different genome-wide scans performed in Finnish families[1] or sib-pairs[2].

STUDY SAMPLE	TRAIT	LOCUS	AUTHORS AND YEAR
FCHL[1]	FCHL	1q21-q23	Pajukanta et al. 1998
	high TGs	10q11	Pajukanta et al. 1999
Low HDL-C[1]	low HDL-C	8q23.1	Soro et al. 2002
		16q23.3	
		20q13.32	
Premature CHD[2]	CHD	2q31	Pajukanta et al. 2000
		Xq24	

TG and HDL-C are highly inversely correlated traits; probably exhibiting at least a partially shared genetic background. To better address this, we analyzed the data of the most promising region of this study (10q11) with a novel QTL association program, which is able to take into account these two QTL traits, TGs and HDL-C, at the same time. In these analyses, we detected evidence for association for quantitative TGs with D10S546, about 7 cM from D10S1772 (p-value of 0.0006). For HDL-C, the association analysis produced a p-value of 0.02 with marker D10S1790, located in the vicinity of

D10S546 (0.0 cM, 889 kb). Furthermore, a combined analysis of quantitative HDL-C and TGs produced a p-value of 0.008 again with marker D10S546 in the association analysis.

Figure 9. Combined study sample of dyslipidemic families.



To summarize the results, we found strong evidence for linkage of the chromosome 10q11 locus with the quantitative HDL-C trait in a combined data analysis, using a variance-component approach (a lod score of 3.3). Furthermore, this region, previously linked to TGs, BMI and obesity (Hager et al. 1998; Pajukanta et al. 1999; Hinney et al. 2000; Ohman et al. 2000; Price et al. 2001), provided evidence of association for both quantitative HDL-C and TG traits. The lowest p-value of 0.0006 was obtained with quantitative TG trait for the marker D10S546. In conclusion, the data suggest that a locus on chromosome 10q11 influences the variation in plasma HDL-C and TG levels in Finnish dyslipidemic families.

4. FINEMAPPING OF THE 1q21 REGION IN FCHL FAMILIES (IV)

The chromosome 1q21 was originally linked to FCHL in Finnish families (Pajukanta et al. 1998). Since then the finding has also been replicated in more heterogeneous populations (Coon et al. 2000; Pei et al. 2000; Allayee et al. 2002). Interestingly, the same region has also been linked to type 2 DM and the metabolic syndrome (Hanson et al. 1998; Elbein et al. 1999; Watanabe et al. 2000; Vionnet et al. 2000; Wiltshire et al. 2001; Hsueh et al. 2003; Langefeld et al. 2004; Xiang et al. 2004). Since FCHL and type 2 DM share several phenotypic features, allelic variants of the same gene may underlie the linkage results obtained. The aim of this study was to identify the underlying FCHL gene on 1q21.

First, a strong positional candidate for human FCHL, the thioredoxin interacting protein (TXNIP) (Pajukanta et al. 2001), was sequenced to identify all possible variants of the gene. All identified 20 SNPs were rare (allele frequencies < 10%) and produced no changes in amino acids. Four TXNIP SNPs was analyzed and, although some evidence for linkage was detected, neither evidence for association or LD, nor evidence for shared haplotypes was found. Thus, the human homolog of the Hyplip1 gene (Castellani et al.

1998), the TXNIP gene, is unlikely to be the causative gene in FCHL, because no statistical evidence emerged in extensive statistical analyses of the identified TXNIP variants or their haplotypes. Furthermore, the human Hyplip1 gene is located about 15 Mb from the peak linkage marker D1S104 in humans, making it a less likely candidate.

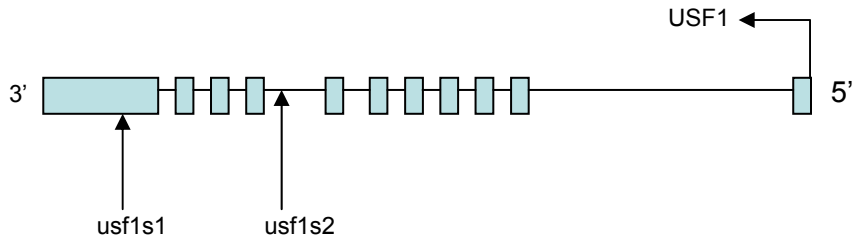
Second, three relevant regional candidate genes were sequenced, the upstream transcription factor 1 (USF1), the retinoid X receptor gamma (RGRG), and APOA2. The SNPs that were identified by sequencing the RXRG and APOA2 genes did not result in missense or nonsense variants, and no evidence for association was observed between the SNPs or their haplotypes and FCHL or TGs. Likewise, none of the SNPs that were identified by sequencing of the USF1 gene resulted in amino acid changes. However, two SNPs, usf1s1 (exon 11) and usf1s2 (intron 7), provided evidence for linkage, producing lod scores of 3.5 and 2.0 for FCHL, and 3.7 and 2.0 for TGs. Combined analysis of these two USF1 SNPs produced some evidence for association for both FCHL (p=0.005) and the TG trait (p=0.008) using the gamete competition test. Usf1s1 and usf1s2 also suggested an association in TG-affected men, and combined analyses of these two SNPs resulted in p-values between 0.02–0.003. Since these SNPs yielded such promising results with the 42 FCHL families genotyped so far, all 60 FCHL families (721 individuals) were genotyped for usf1s1 and usf1s2.

In the analyses of the two USF1 SNPs both the FCHL and TG traits showed evidence for linkage and association (p=0.00002 and 0.00006). The strongest evidence for association was seen in TG-affected males (p=0.0000009), suggesting some sex-specific effect especially concerning the TG trait. Gender-specific differences are typical for complex traits and both genetic and environmental factors are likely to contribute to these differences. Male sex is a well-known risk factor for CHD, and interestingly, recent studies also suggest gender-specific differences in the dyslipidemic phenotypes in FCHL (Soro et al. 2003).

As mentioned above, the analysis of the two USF1 SNPs, usf1s1 and usf1s2 (Figure 10) provided strong evidence for association. The segregating haplotype in all these analyses was 1-1 (1 indicates the common allele). A total of 15 SNPs were genotyped for the USF1 region. Evidence for association was observed with several SNPs. In the TG-affected males the association extended to the adjacent F11R gene over a 46 kb region, whereas the haplotype analyses in all FCHL- and TG-affected family members restricted the associated region to 1239 bp within the USF1 gene. In addition, haplotype analysis revealed that the 1-1 haplotype of the usf1s1-2 was transmitted significantly more to the TG-affected individuals (P=0.00003), especially in TG-affected males (P=0.0000009).

In TG-affected men, 6 SNPs in the F11R-USF1 region showed evidence for association, and 5 of these were in strong LD. F11R, also known as JAM1, Junctional adhesion molecule 1, plays an important role in the regulation of tight junction assembly in epithelia. In addition, F11R is involved in the pathogenesis of viral infections and it serves as an integrin ligand. However, the extent of LD in FCHL alleles of both sexes covers only the USF1 gene and the known functions of F11R make it a less likely candidate for FCHL as compared with USF1.

Figure 10. Upstream transcription factor 1 protein is encoded by the 6.74 kb USF1 gene, which maps on chromosome 1q22-q23. Two associated USF1 SNPs are shown in this figure: usf1s1 is located in exon 11 and usf1s2 in intron 7.



To summarize the genetic data, evidence for association, linkage and shared haplotypes was found between USF1 and FCHL. The evidence for association was strongest in TG-affected men. In all the affected FCHL family members, evidence for association (P=0.00002) and LD was restricted to the USF1 gene. However, in the TG-affected men, evidence for association (P=0.0000009) and LD extends at least 46 kb within the F11R-USF1 region.

Interestingly, preliminary functional data obtained with microarray analysis supported the genetic data: the USF1 risk haplotype had an effect on the expression profiles of fat biopsies. Expression profiles of fat biopsies from affected FCHL family members carrying the 1-1 susceptibility haplotype of USF1 revealed expression differences when compared to affected FCHL family members without this haplotype and homozygous for the putative protective haplotype 2-2. Applying highly stringent criteria, 25 genes were identified as upregulated and 73 genes as downregulated in the risk haplotype carriers. Genes with expression differences included APOE, PLTP, MSP1 and ALOX5. However, no differences in the steady state USF1 expression levels could be observed between FCHL patients ascertained for the risk haplotype, suggesting that the potential functional significance of the associated USF1 allele is not delivered via a direct effect on transcription of this gene in adipose tissue.

To lend biological relevance to the array findings, the lists of differentially expressed genes were examined for any over-representation of functional classes. Only three classes were significantly over-represented among the upregulated genes in the risk haplotype carriers. These were primarily genes involved in fat metabolism. Interestingly, a prominent downregulation of immune-response genes was also observed.

A novel putative Cis-acting regulatory element was identified in intron 7 of USF1, in the immediate vicinity of the USF1 risk haplotype. The genomic sequence flanking the risk haplotype was investigated for potential functional domains, and a 60-bp sequence element present in 91 human genes was identified. The SNP usf1s2, forming part of the risk haplotype, resides adjacent to a 306-bp Alu repeat. Two parts of this Alu repeat show sequence similarity with the mouse B1 repeat. When blasted against the mouse sequence databases, these two parts of the Alu sequence identify numerous mouse ESTs, due to the B1 element located in the untranslated region of the mouse mRNA. A total of 91 human genes, including USF1, have this 60-bp part of Alu located either on the coding strand or

on the opposite strand. The 60-bp part is highly conserved, and found from human to worm but not in Drosophila melanogaster or in Saccharomyces cerevisiae.

Interestingly, the region in intron 7 of USF1 containing the 60-bp conserved sequence and the usf1s2 SNP showed transcription efficiency. A SEAP reporter assay demonstrated that this 60-bp element has an effect on transcription in vitro in the forward orientation. The reverse orientation resulted in transcription efficiency comparable to that of the negative control, implying a Cis-acting regulator rather than a direction-independent enhancer element. These data suggest a putative regulatory element in the immediate vicinity of the USF1 risk haplotype.

DISCUSSION

The aim of this thesis was to identify disease loci and to search for susceptibility genes for two common atherosclerosis related lipid disorders, low HDL-C and FCHL. First, we detected linkage for low HDL-C near two candidate genes, APOA2 and the APOA1C3A4 gene cluster (I). Second, in a genome-wide scan, three novel loci for low HDL-C were identified, on 8q23, 16q24.1-24.2, and 20q13.11 (II). Third, fine mapping of the previous loci provided more evidence for the importance of the 10q11 region: an association was found between HDL-C and TG levels and 10q11 (III). Fourth, a first susceptibility gene for FCHL, USF1, was identified on 1q21 (IV).

Multiple candidate genes have been suggested to affect plasma HDL-C levels and genes related to FCHL (Tables 8 and 9) and several loci for these lipid traits have been detected (Tables 10 and 11). Typically in complex disorders, the first identification of a genetic polymorphism to be associated with a disease does not automatically lead to replication of the original findings in other study samples and in different populations. The problem in replicating the results may be due to several factors, including phenotypic heterogeneity, inadequate statistical approaches, an insufficient study sample size, or a type 1 error. The failure to replicate a previous positive linkage finding may also be explained by different ethnicity, if the original linked variation is population specific. In future, new statistical methods are needed to better model the complexity caused by several interacting genes and environmental factors.

Replication of the original finding in other study samples and in different populations provides further evidence for the impact of the gene or locus to the trait. Interestingly, some of the results of this thesis study were replications of the previous results or have since then been replicated. Before the detection of linkage of a low HDL-C trait to the APOA2 locus (I), this chromosomal region on 1q21-q23 was linked to FCHL in Finnish (Pajukanta et al. 1998) as well as in some other FCHL families (Coon et al. 2000; Pei et al. 2000). In addition to Finnish low HDL-C families (I), evidence for linkage to near the APOA1C3A4 gene cluster has also been demonstrated in 105 large Utah pedigrees with hypoalphalipoproteinemia (Kort et al. 2000).

Involvement of 8q23 (II) in the regulation of serum HDL levels was earlier shown in randomly ascertained Mexican-American families (Almasy et al. 1999). Further evidence of involvement of a locus on 16q in the regulation of plasma HDL-C levels (II) has been obtained in Mexican Americans (Mahaney et al. 2003) and recently in a combined data analysis of Finnish and Dutch FCHL families (Pajukanta et al. 2003). The 20q13.32 region, which was linked to TGs and HDL-C (II, III), has shown evidence for linkage to type 2 DM and obesity in several studies and in different populations (Bowden et al. 1997; Ji et al. 1997; Zouali et al. 1997; Ghosh et al. 1999; Lee et al. 1999; Ghosh et al. 2000; Mohlke et al. 2001).

The clustering of linkage peaks for component traits of the metabolic syndrome also occur in other chromosomal regions. For example, linkage between 10q11 (III) and obesity has been detected (Hager et al. 1998; Hinney et al. 2000; Price et al. 2001). Likewise, 1q21-q23 is not only linked to FCHL in multiple studies, but also to type 2 DM and the metabolic syndrome (Table 12) (Pajukanta et al. 1998)(Hanson et al. 1998; Elbein

et al. 1999; Coon et al. 2000; Pei et al. 2000; Watanabe et al. 2000; Vionnet et al. 2000; Wiltshire et al. 2001; Allayee et al. 2002; Hsueh et al. 2003; Langefeld et al. 2004; Xiang et al. 2004). Taken together, these findings suggest that one or more genes in this particular chromosomal region may predispose to FCHL, type 2 DM, and the metabolic syndrome, and thus, also to premature CHD. The clustering of linkages in the same chromosomal area is consistent with the fact that many of the critical metabolic features of FCHL (e.g. hypertriglyceridemia and insulin resistance) also represent trait components of type 2 DM and the metabolic syndrome.

The goal in disease gene mapping is to identify new disease genes in order to understand the underlying biological mechanisms and to offer tools for the development of molecular diagnostics, and preventive medicine, as well as drug and possible gene therapy. In a study of FCHL, we have now reached the first step by characterizing a putative susceptibility gene underlying this lipid disorder (IV). Currently we cannot positively confirm one single associated causative variant in the Finnish FCHL families, but rather we identified several associated SNPs in tight LD and a common SNP haplotype, segregating predominantly to affected family members.

FCHL is a typical complex trait, in which several genes, environmental factors and their interactions underlie the disease phenotype. Variants involved in complex disease are likely to be located in promoters and other regulatory regions of genes, rather than in the coding regions. Polymorphisms in the regulatory regions may be particularly relevant in chronic metabolic diseases, such as FCHL, leading to a gradual accumulation of damage over many years before reaching a critical threshold. It is thus possible that, in complex diseases, variations in regulatory sequences cause slight differences in message levels, timing and tissue specificity of gene expression, and protein stability, that lead to subtle quantitative differences in phenotype (Mackay 2001). Accordingly, our finding identifying a novel regulatory element within intron 7 of USF1 may turn out to exemplify this by possibly providing a novel promoter for a mini-USF1 protein lacking the transactivation domain (Shoulders 2004).

To conclude, several loci affecting plasma HDL-C and TGs levels have been identified in this thesis (APOA2 and APOA1C3A4 gene cluster loci, 8q23, 16q24.1-24.2, and 20q13.11 and 10q11) (I-III). Most interestingly, one of the studies led to the characterization of a new susceptibility gene for FCHL, USF1 (IV). The strong association between the USF1 gene and FCHL suggests that USF1 contributes to the susceptibility to FCHL. Because the USF1 gene encodes a transcription factor known to regulate several genes participating in glucose and lipid metabolism (for refs. see below), it is potentially the missing link between FCHL and type 2 DM. USF1 may form a prime candidate in the 1q21-23 region for increasing the susceptibility to FCHL, type 2 DM, and the metabolic syndrome. Moreover, USF1 might play an important role in low HDL-C, because it resides on chromosome 1 in the vicinity of APOA2 and the low HDL-C trait was linked to that region (I). Although additional studies are needed to clarify the mechanisms of the connection between USF1 alleles and the FCHL phenotype, our results provide novel insights into the genetic background of FCHL and low HDL-C, the most common familial lipid disorders predisposing to CHD.

USF1

USF1 is a transcription factor of the basic helix-loop-helix leucine zipper family. It forms homo- and heterodimers (with USF2) and recognizes a CACGTG motif termed E box (Casado et al. 1999; Ribeiro et al. 1999), resulting in activation of gene transcription (Chen et al. 2001) and enhanced expression in response to various stimuli, such as glucose and dietary carbohydrates (Nowak et al. 2004). USF1 is known to regulate several genes participating in glucose and lipid metabolism. In the liver, USF1 regulates the expression of several genes, including fatty acid synthase (FAS) (Casado et al. 1999), APOA2 (Ribeiro et al. 1999) APOC3 (Pastier et al. 2002), APOE (Salero et al. 2003), HL (Botma et al. 2001), and ABCA1 (Yang et al. 2002). In addition, the gene expression of an important factor in hepatic glucose sensing, glucokinase, is regulated by USF1 (Iynedjian 1998). In the pancreas, USF1 controls the expression of insulin (Read et al. 1993), the glucagon receptor (Portois et al. 2002), and the islet-specific glucose-6-phosphatase catalytic-subunit-related gene (Martin et al. 2003a). In adipose tissue, USF1 mediates the insulin-responsive expression of FAS (Wang and Sul 1995; Wang and Sul 1997), the glucose-regulated expression of hormone sensitive lipase (Smih et al. 2002), as well as the gene expression of acetyl CoA carboxylase (Travers et al. 2001). On the basis of these studies, USF1 seems to play a central role in the regulation of several of the key genes of lipid and glucose metabolism.

In the USF knock out mice, the normal transcriptional response of liver genes to glucose seemed to require the presence of either USF1/USF2 heterodimers or USF2 homodimers (Vallet et al. 1998). In these mice, induction of the FAS gene was severely delayed by refeeding a carbohydrate-rich diet, suggesting that USF transactivators, especially USF1/USF2 heterodimers, are essential for sustaining the dietary induction of the FAS gene in the liver (Casado et al. 1999). USF1/USF2 is also capable of activating the transcription of the gene encoding renin, which is an important regulator of the systemic blood pressure (Pan et al. 2001). It has also been proposed that USF genes may play an important role in the differentiation capability of vascular smooth muscle cells, because USF factors modulate the expression of genes that are acutely regulated by the differentiation status of the smooth muscle cells of the vasculature (Chen et al. 2001).

In our study (IV), a specific haplotype of USF1 was associated with FCHL. Interestingly, Putt and colleagues studied three intronic USF1 SNPs in more than 800 healthy men, and reported that the 475C/T-1738C/T haplotype influences the glucose levels during the oral glucose tolerance test (Putt et al. 2004). Furthermore, they found case:control heterogeneity in the interaction between USF1 SNPs genotypes and BMI on fasting LDL and glucose.

It has been suggested that the novel regulatory element identified within intron 7 of USF1 may provide a novel promoter for a mini-USF1 protein lacking the transactivation domain (Shoulders 2004). The putative promoter identified (IV) might operate in vivo to downregulate USF1 activity, because mini-USF proteins have been shown to act in vitro as transdominant inhibitors (Lefrancois-Martinez et al. 1995; Viollet et al. 1996). This theory proposes that intronic variants affecting splice sites or transcription factor binding sites may be of significant importance in complex traits, and this very interesting possibility remains to be investigated.

CONCLUDING REMARKS

In the course of this study, methods for mapping and identifying genes for complex traits have developed tremendously. Especially, the sequence of the human genome produced by the HGP and the genetic databases publicly available for everyone in the World Wide Web are valuable tools for researchers, and are rapidly changing the course of gene hunting from the old methods to a new direction. The increasing numbers of SNPs will facilitate the construction of haplotypes in different chromosomal regions and different populations. Knowledge of the haplotype structures will help to evaluate the association between a genetic marker and a trait in LD mapping. It might also help us to solve which type of populations would be ideal for determining the genetic background of complex disorders.

Having the anatomy of the human genome in front of us offers magnificent opportunities for dissection of the molecular basis of human diseases. Now, in the genomic era, biomedical research is facing changes in its methods and strategies. Structural genomics will focus on functional genomics and proteomics. As we turn from monogenic disorders more and more to dissection of multifactorial disorders, we are monitoring susceptibility rather than causality where analysis of one gene is changing to analysis of multiple genes or gene families, pathways, and systems. Other changes include shifting from research on gene action to gene regulation and from etiology (specific mutation) to pathogenesis (the mechanism of the disease).

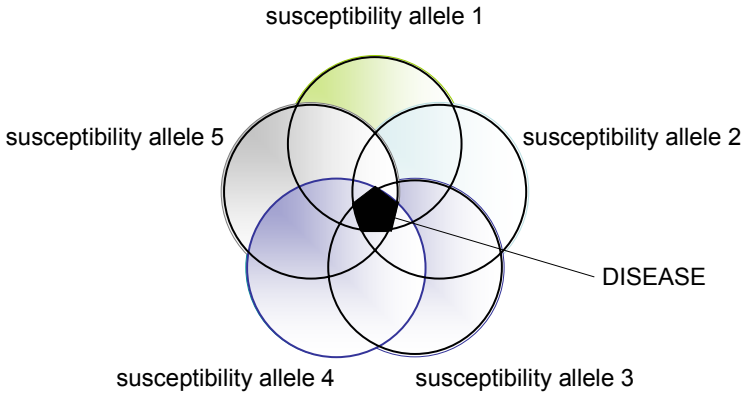
Although we have now gained a huge amount of new information about genomics, we do not yet have good guidelines or experience on how to use this knowledge effectively. To get the most out of this new information, efficient collaboration between research groups, data sharing, and pooled data analyses are required in the genomic era. In addition, collaborations among clinicians, epidemiologists, geneticists, statisticians and other experts have become increasingly important and are needed to solve the complex genetic background of multifactorial traits.

Although complex traits are clinically important and common in the population, relatively little is known about the nature of the genetic variation underlying complex diseases. Moreover, most of our knowledge about the genetics of common diseases relates to rare families segregating high-risk alleles (Botstein and Risch 2003), which are generally very infrequent in the population and therefore explain relatively little of overall disease prevalence. In contrast, an important contribution of common variants to the susceptibility to common diseases has been suggested: there are probably many common variants in the human genome with modest but real effects on complex disease risk (Figure 11) (Lohmueller et al. 2003). Likewise, the distribution of quantitative traits in the population is presumed to reflect the cumulative contribution of multiple common DNA sequence variants, each having a small effect (Figure 11).

For complex disorders many chromosomal loci have been identified, but only a small proportion of these findings have led to actual identification of the underlying gene. How can we improve gene identification strategies for complex traits? The tools for the genetic dissection of such traits are improving, and the new methods may even help to better reveal the minor effects of multiple genes in complex disorders. For instance, microarray

methodology provides valuable information about new metabolic pathways, helps to find the genes that are important in disease pathogenesis, and may even show which genes and alleles cause significant risks in the general population. Genome wide association studies, dense SNP maps, and haploblocks of the HapMap-project are examples of tools that most likely will facilitate identification of the genetic background of complex traits.

Figure 11.
A schematic overview of how disease susceptibility alleles, which are common alleles in the population, predispose to a disease. Risk alleles of many different susceptibility genes together with environmental factors cause the disease.



The HGP have made a tremendous contribution to the positional cloning of complex traits by providing a complete catalogue of all the genes residing in a relevant region. Now researchers can go to a variety of websites (such as the National Center for Biotechnology Information, NCBI, http://www.ncbi.nih.gov or UCSC Human Genome Browser http://www.genome.ucsc.edu) to find tens of thousands of well-characterized genes and to start systematically evaluating candidate genes for the linked chromosomal region instead of tedious original positional cloning.

The most attractive finding of this thesis is the identification of, a susceptibility gene for FCHL in Finnish families, USF1. Nevertheless, this study also exemplifies many problems encountered in the genetic research of complex traits. Probably the most typical question is how to prove that noncoding variants that are associated with a trait really predispose to disease? It seems that not only one SNP but rather a group of adjacent SNPs residing in a relatively wide chromosomal region are associated with disease due to strong LD between these SNPs. So which one is *the* variant and *the* gene that causes susceptibility to disease?

To understand the mechanisms of USF1 in FCHL and to clarify whether USF1 also plays a role in the metabolic syndrome, additional studies are needed. The future plans should include several different approaches. First of all, the challenge ahead is to confirm our promising genetic data through replication. Investigation of the associated USF1 variants in the Dutch FCHL families and in large epidemiological population samples such as the Finnish cohort "Finrisk 1992" is currently ongoing. A recent study by Putt and colleagues offered further evidence that USF1 influences glucose and lipid homeostasis, even in healthy men (Putt et al. 2004).

Second, functional studies of the associated USF1 SNPs, especially usf1s1 and usf1s2, are warranted to determine the roles of the different isoforms of USF1 in the regulation of genes important for lipid and glucose homeostasis. For example, functional proof of regulatory sequence variations may be achieved by techniques of cell biology or bacterial artificial chromosome transgenesis (Symula et al. 1999).

Third, further microarray studies in a larger study sample are needed in order to be able to confirm our preliminary results. The microarray studies should be carried out not only in fat tissue samples but also in different tissues, for instance in muscle tissue obtained from muscle biopsies.

Fourth, animal studies could help us to better understand the underlying molecular mechanism of the USF1 gene in FCHL. For example a transgenic USF1 mouse model could offer a useful tool for that.

Fifth, in order to analyze the role of the USF1 gene in type 2 DM, investigations of the associated USF1 variants in type 2 DM study samples are necessary. In summary, further studies in large epidemiological samples of the Finnish population, and in other well-defined FCHL samples from more mixed populations, as well as in type 2 DM families, will help to clarify the contribution of USF1 not only to FCHL but also to other FCHL-associated phenotypes, type 2 DM and the metabolic syndrome.

FCHL, the most common inherited disorder of abnormal blood lipid levels, is a highly atherogenic multifactorial disorder. As in other complex traits, multiple susceptibility and modifying genes are believed to play a role in the pathogenesis of FCHL. The identified USF1 gene forms a promising candidate gene for FCHL and is also an encouraging example of a gene detected for a complex multifactorial lipid trait. However, the major task in future projects is not only to confirm the role of USF1 but also to identify the other genes associated with FCHL.

In Finnish low HDL-C families, gene hunting will continue. The impact of the USF1 gene in low HDL-C families needs to be investigated. In addition, the role of the APOA2 gene and APOA1C3A4A5 gene cluster in low HDL-C requires additional studies. Future plans include further investigation of the loci linked to the low HDL-C trait found in our previous study (8q23, 16q24.1-24.2 and 20q13.11), as well as sequencing of the promising candidate genes located on 10q11, a region associated with HDL-C and TG levels. The idea of the haplotype blocks capturing most of the genetic variation in a region could be employed as a new strategy to identify the gene for HDL-C on 10q11. If the 10q

region fits the block theory well, this novel approach could significantly decrease the number of SNPs that need to be analysed in order to find the associated gene.

The genomic era offers us great opportunities and only the future will show us, how well we will be able to take advantage of the huge amount of information now available about the human genome. In the end, the ultimate goal is to translate genomic research into health applications, i.e. to harness genetics to help find better ways of diagnosing, treating and preventing disease, and finally, to improve health.

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